CHEMICAL GENETIC REGULATION OF MALONYL-COA DECARBOXYLASE IN SKELETAL MUSCLE INSULIN RESISTANCE

by
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A dissertation submitted to Johns Hopkins University in conformity with the requirements for the degree of Doctor of Philosophy

Baltimore, Maryland
December 2013

ABSTRACT

The rise in obesity and its association with insulin resistance is attributed to excess caloric consumption and sedentary living. Skeletal muscle has been demonstrated to be the primary tissue driving insulin resistance before beta-cell failure in type 2 diabetes, therefore it is a target for anti-diabetic drugs. The mechanisms leading to the development of skeletal muscle insulin resistance in obesity and type 2 diabetes remains unresolved, but defects in skeletal muscle fatty acid oxidation have been implicated in the etiology. Malonyl-CoA decarboxylase (MCD) has been a target of investigation because it regulates the concentration of malonyl-CoA, a central metabolite in fatty acid biochemistry. The purpose of this thesis was to determine the *in vivo* role of MCD in skeletal muscle of obese and insulin resistant mouse models. We generated mice expressing a muscle specific transgene for MCD (Tg $fMCD^{Skel}$) regulated posttranslationally by the small molecule, Shield-1. Tg $fMCD^{Skel}$ and control mice were placed on either a high fat or low fat diet for 3.5 months. Obese and glucose intolerant as well as lean control Tg $fMCD^{Skel}$ and nontransgenic control mice were treated with Shield-1 and changes in their body weight and insulin sensitivity upon induction of MCD were determined. Obese Tg $fMCD^{Skel}$ mice had no improvement in body weight or glycemic control after 2 weeks of MCD induction. Obese Tg $fMCD^{Skel}$ and control mice were then subjected to an acute Shield-1 treatment prior to an *in vivo* insulin stimulation. Compared to obese nontransgenic controls, Tg $fMCD^{Skel}$ mice had decreased AKT signaling in the skeletal muscle. In addition, oxidative metabolism genes were down-regulated at the transcriptional and protein level compared to obese controls. The suppression of mitochondrial oxidative genes suggests an unexpected redundant and

metabolite driven regulation of gene expression through the transcriptional regulator, PPARα. Together, these data indicate that induction of MCD leads to decreased fatty acid oxidation gene expression, impairs skeletal muscle insulin sensitivity, and implicates PPARα in a fatty acid induced down regulation of oxidative genes *in vivo* to regulate the muscle's adaptive response to diet induced obesity and insulin resistance.

Thesis Advisor: Dr. Michael Wolfgang
Thesis Reader: Dr. Paul Watikins

ACKNOWLEDGEMENT

I am grateful to my mentor, Dr. Michael Wolfgang for his guidance, support, and dedication towards my successes as a doctoral student. I am indebted to Dr. Laura Robles and Dr. Thomas Landefeld for introducing me to basic science research during my studies at CSUDH. I am indebted to my undergraduate research mentor, Dr. Amiya Sinha Hikim for supporting my growth as a young scientist in his lab at Harbor-UCLA. I am appreciative to my friends, Dr. Rafael Luna and Dr. Jodel Girard because their mentorship, encouragement, and enthusiasm were a great source of inspiration to help me get through the hardships of graduate school. I am thankful to Dr. Natasha Zachara and Dr. Guang William Wong for providing me with encouragement, support, and feedback on my thesis projects.

I thank my friends in CMOR and other labs for making my work environment wonderfully wacky and fun: Marcus Seldin, Jessie Wilcox, Jieun Lee, Caitlyn Bowmen, Stephanie Tan, Zeke Wei, Kamau Fahie, and Courtney Akitake. I also thank my friends outside of lab for their love and support: Ana Rita Nunes, Nisha Bhat, Mindy Kim Graham, Isabel Casimiro, Julie Garchow, Denise Torres, and Ederlen Casillas.

Finally, I am deeply indebted to my loving parents, Eva and Ramon Rodriguez. What I achieve in life could not have happened without their support. I thank them for the sacrifices they made to provide me with a better life in the US. I am grateful to my husband, Colin Smith for his endless love and support throughout my graduate studies. I thank my in-laws, Sharon and Ron Smith for their encouragement, love and support.

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CHAPTER I: Overview

The Obesity, Insulin Resistance, and Type 2 Diabetes Epidemic

Obesity

Excessive adiposity is a major health problem in the United States where > 60% of adults are overweight and greater than one-third are clinically obese as defined as a Body Mass Index of >30. Obesity is a major risk factor for insulin resistance, Type 2 Diabetes, cardiovascular and cerebrovascular disease, hypertension, stroke, and other chronic disorders that lead to morbidity and mortality (1-3). Beyond the accumulation of fat in adipose tissue, hyperlipidemia in obese and diabetic patients is a major contributing factor for stroke and heart attack. The magnitude of obesity related vascular disease is staggering. Obesity related disease is predicted to become the leading cause of preventable death over the next decade. Lifestyle interventions used to promote weight loss are met with high rates of recidivism (4). Individuals regain one third of their lost weight in the first year after treatment and in some cases continue weight gain in their life time. Researchers and clinicians struggle to find useful strategies for successful weight loss maintenance, thus adding emphasis to the development of novel approaches to weight loss therapy.

Obesity is the result of energy imbalance. Thus, when caloric intake exceeds expenditure, metabolic flux is directed into energy reserves, primarily triglyceride in adipose tissue. Conversely, when caloric expenditure exceeds intake, these reserves are mobilized to provide physiological fuel for peripheral tissues. To alter body weight, in particular adiposity, either energy intake and/or expenditure must be altered. The brain and peripheral tissues play an important role in regulating the homeostatic mechanisms

that control energy balance and therefore, the magnitude of body fat reserves (5-9). The crosstalk mediated through multiple organ systems work to regulate complex metabolic processes by coordinating their actions through secreted hormones (e.g., insulin, glucagon, leptin, adiponectin, FGF21) to maintain the integrated control of whole-body metabolism **(Figure 1).** Disruption of these hormone-mediated metabolic circuits frequently results in disrupted energy metabolism and related pathologies.

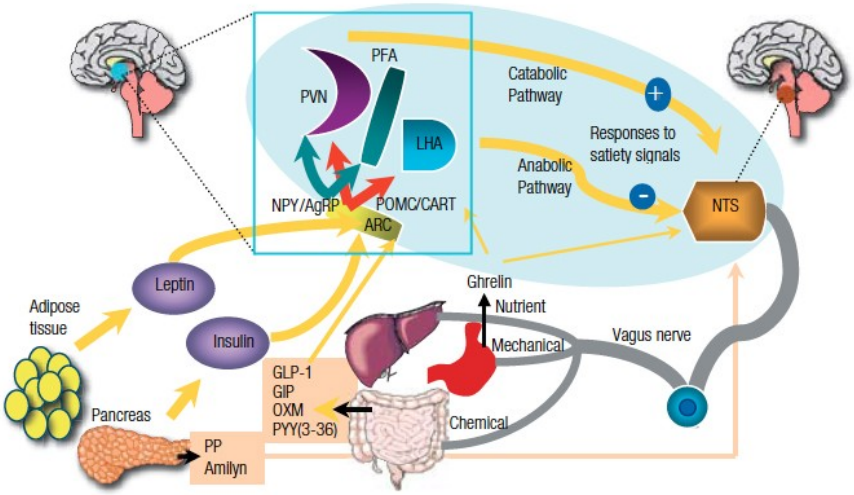


Figure 1-1: **Integrated control of Metabolism**. Signals such as leptin and insulin are secreted in proportion to the size of the fat mass and circulate in the blood. They enter the brain and act at the level of the hypothalamus. Neuroendocrine signals from the stomach, the gastrointestinal system and the liver are sent to the hindbrain, providing information about the food that is eaten:its taste and chemical content, and how much the stomach is distended. Arq Bras Endocrinol Metab. 2009;52(2):271-280.

Obesity Induced Insulin Resistance and Type -2-Diabetes

Impaired activity of the insulin signaling pathway, eventually resulting in insulin resistance can be caused by genetic and/or acquired through poor lifestyle and environmental factors (10, 11). The obesity epidemic is linked with the rise of inulin resistance and type 2 diabetes throughout in the United States **(Figure 2)**, most commonly acquired through a sedentary lifestyle, increased food consumption and aging (10-12)**.** While genetic causes or predispositions toward insulin resistance within populations have been suggested, they are poorly understood (13). Environmental factors, such as excessive adiposity and sedentary lifestyles, mentioned above are contributing factors for the rise in obesity related insulin resistance (14). The mechanisms of insulin resistance are not clear, but are linked to mitochondrial deficiency

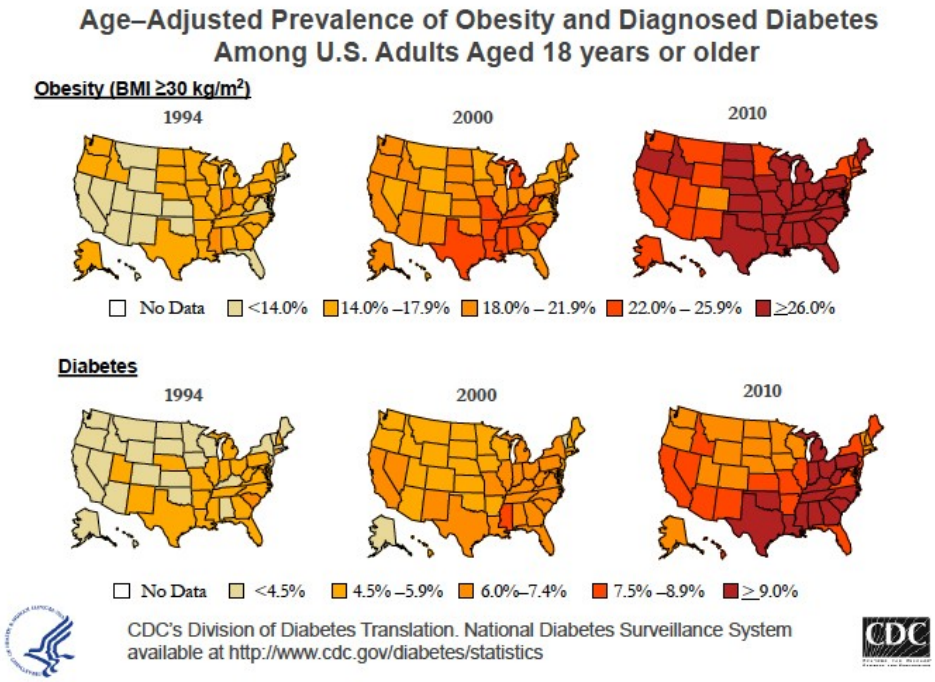


Figure 1-2. Maps of Diagnosed Diabetes and Obesity in 1994, 2000, and 2010 (2011). Source: CDC's Division of Diabetes Translations. National Diabetes Surveillance System. Source: *http://www/cdc.gov/diabetes/statistics.*

inflammation, ER stress, lipid storage in non-adipose tissues, and cancer (12, 15, 16). Insulin resistance is defined as the failure of insulin sensitive target tissues to respond to the effects of circulating insulin. Impaired insulin sensitivity occurs in the skeletal muscle, liver and adipose tissue. (17).

Although they share similar mechanisms in development, insulin resistance and type-2-diabetes are different. Type 2 diabetes involves pancreatic β-cell failure due to the overwhelming demand for insulin. Increased insulin synthesis and secretion from β-cells leads to an expansion of the β-cell mass until β-cells can no longer compensate for decreased tissue insulin sensitivity. The mechanisms involved in the transition from an insulin resistant state to overt diabetes remains unclear. Type 2 diabetes is projected to afflict 366 million people worldwide by 2020 based on 2004 estimates (14)**.** Updated figures collected by the International Diabetes Federation show this estimate has been surpassed in 2012 (**Figure 3).** The best preventative therapy is to decrease body weight and increase healthy lifestyle habits; however, the general public approaches lifestyle interventions through diet and exercise with resistance and failure. Typical, lifestyle interventions have high recidivism rates, with patients regaining one third of their weight after the first year and, at times continuing (4). Pharmacological interventions (e.g. orlistat and sibutramine) are often of limited effectiveness at promoting substantial weight loss (5-10%) (4). As the rates of obesity and insulin resistance continue to increase, understanding the mechanisms involved in the development of these and other forms of metabolic dysfunction remains of the utmost importance.

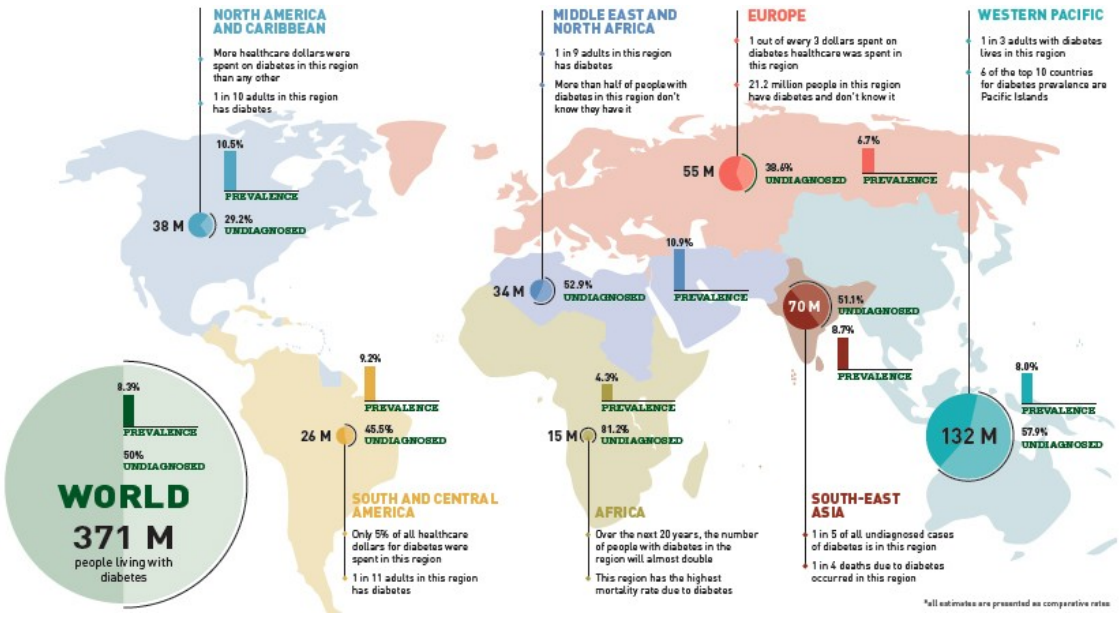


Figure 1-3. International Diabetes Atlas, 5th Edition, 2012 Update. The Latest Global and Regional Diabetes Statistics. Source: *International Diabetes Federation http://www.idf.org/diabetesatlas*

Insulin and Glucagon

Hormones are important for regulating biochemical switches in tissues in response to changing nutritional states. Glucose homeostasis in maintained by the pancreatic hormones: insulin and glucagon. They work in a reciprocal manner to affect circulating blood glucose concentration. Insulin is secreted in response to elevating high blood sugar, obtained after eating a meal. Insulin reduces blood glucose by promoting glucose uptake by the muscle, red blood cells, liver, and adipose. Glycogen, a polymer of glucose, is synthesized in the liver and muscle during the fed response and is the short - term energy storage derived from sugars. The liver and adipose tissue shuttle glucose towards the production of fatty acid biosynthesis, promoting fat storage. In the fasted state ([high glucagon: low insulin]), glucagon is secreted by the pancreatic alpha cells to

increase blood glucose levels by stimulating the conversion of glycogen to glucose in the liver and muscle, while breaking down fat and protein into glucose, which is secreted into the blood to maintain blood glucose levels.

Insulin Signaling

Insulin regulates efficient energy storage by partitioning dietary substrates towards anabolic metabolism in order to make energy available for use when insulin levels are low. Following the ingestion of a meal, insulin is released from the pancreatic islet β-cells and activates its receptor on fat, muscle, and liver cells to increase glucose, fatty acid, amino acid uptake and stored while simultaneously blocking the breakdown of these stored energy reserves. Insulin action is mediated through the activation of the insulin signaling pathways **(Figure 4)** (18**)**. Insulin binds to the insulin receptor (IR), which leads to the autophosphorylation of the IR and causes the recruitment and phoshphorylation of multiple scaffolding proteins, including the insulin receptor substrate (IRS) proteins, Src-homology-2containing protein (SHC), and the c-Cbl (CBl) protooncogene. Phosphorylation of the IRS proteins, such as IRS1 and IRS2, leads to the recruitment of the p85 regulatory domain of phosphatidylinositol 3-kinase (PI3K) and its association with the p110 catalytic domain of PI3K (18). Activated PI3K converts phosphatidylinositol-4,5-bisphosphate (PIP_2) to phosphatidylinositol-3,4,5-triphospahte (PIP_3). PIP_3 elicits additional signaling by providing binding sites to the pleckstrin homology (PH) domains of downstream kinases, such as phosphoinositide-dependent protein kinase-1 (PDK-1), PDK-2, and protein kinase B (Akt). Activation of the Akt kinase leads to the phosphorylation of downstream targets, such as glycogen synthase

kinase -3 (GSK-3) and AS160 Rab GTPase activating protein (GAP) (19). AS160 interacts with small GTPase RAB10 to translocate glucose transporter-4 (GLUT-4) containing vesicles to the cell surface. The culmination of this insulin signaling cascade causes increased glucose uptake and storage in the form of glycogen.

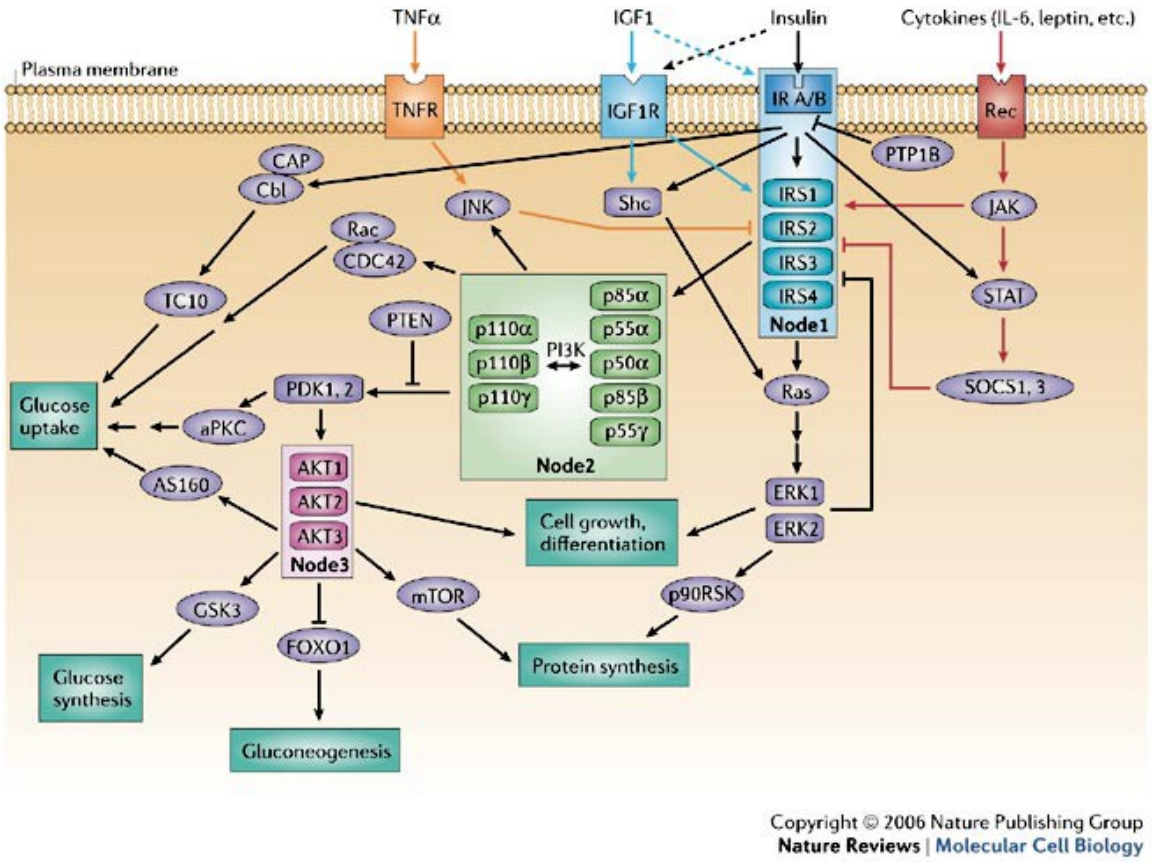


Figure 1-4. **Critical Nodes of Insulin Signaling**. **Critical nodes form an important part of the signaling network that functions downstream of the insulin receptor (IR) (black arrows) and the insulin growth factor-1 receptor (IGF1R) (blue arrows).** Signalling pathways that are activated by cytokines such as tumour necrosis factor-α (TNFα), interleukin-6 (IL-6), and leptin interfere with insulin signalling through crosstalk (orange and red arrows). Three important nodes in the insulin pathway are the IR, the IR substrates (IRS) 1–4 (light blue box), the phosphatidylinositol 3-kinase (PI3K) with its several regulatory and catalytic subunits (light green box), and the three AKT/protein kinase B (PKB) isoforms (pink box). Downstream or intermediate effectors, as well as modulators, of these critical nodes include atypical protein kinase C (aPKC), Akt substrate of 160 kDa (AS160), Cas-Br-M (murine) ecotropic retroviral transforming sequence homologue (Cbl), Cbl-associated protein (CAP), cell-division cycle 42 (CDC42), extracellular signal-regulated kinase 1 and 2 (ERK1 and ERK2), forkhead box O1 (FOXO1), glycogen synthase kinase 3 (GSK3), Janus kinase (JAK), c-Jun-N-terminal kinase (JNK), mammalian target of rapamycin (mTOR), p90 ribosomal protein S6 kinase (p90RSK), phosphoinositide-dependent kinase 1 and 2 (PDK1 and 2), phosphatase and tensin homologue (PTEN), protein tyrosine phosphatase-1B (PTP1B), Ras, Rac, Src-homology-2-containing protein (Shc), suppressor of cytokine signalling (SOCS), signal transducer and activator of transcription (STAT), and Ras homologue gene family, member Q (ARHQ; also called TC10). Dashed arrows represent an activation process with less intensity. *Nature Reviews Molecular Cell Biology 7, 85-96 (February 2006).*

Metabolism of substrates for energy usage: β-Oxidation

Degradation of fatty acids by Beta-oxidation is a multi strep process that occurs in most tissues, including the liver, cardiac muscle, pancreas, skeletal muscle, and brown adipose tissue at different rates. It is the process by which organisms utilizes excess energy from fatty acids and triglycerides. Oxidation rates change in response to a variety of stimuli including fasting, re-feeding, exercise, and disease (20-24) The oxidation of long-chain fatty acyl-CoA begins with the transport of the fatty acid across the mitochondrial membrane by the aid of the carnitine shuttle enzymes: CPT1, CACT, and CPT2 **(Figure 5).** Next, oxidation of long chain fatty acids proceeds by 4 enzymatic steps in the mitochondrial matrix **(Figure 5).** In the first step, acyl-CoA dehydrogenase dehydrogenates the long chain fatty acyl-CoA. In the second step, trans-Δ^2enoyl-coA hydratase hydrates the intermediate at the double bond to form L-3-hydroxyl-CoA. In the third step, hydroxyl-acyl-CoA dehydrogenase oxidizes L-3-hydroxyl-CoA to form 3-ketoacyl-CoA. In the last step, 3-ketoacyl-CoA thiolase cleaves 3-ketoacyl-CoA to form acetyl-CoA and a fatty acyl-CoA that was shortened by two carbons and an acetyl-CoA. In summary, each round of oxidation produces acetyl-CoA and a shortened 2-carbon fatty acyl-CoA product that re-enters the 4-step cycle until the fatty acyl-CoA species is completely oxidized into acetyl-CoA. Acetyl-CoA enters the TCA cycle for further conversion and produces CO_2. The number of acetyl-CoA produced depends on the carbon length of the fatty acid being oxidized. This process produces NADH and FADH2. One FADH2 is produced in step 1 and NADH in the third step. NADH and FADH2 is used by the electron transport chain to produce ATP for energy use.

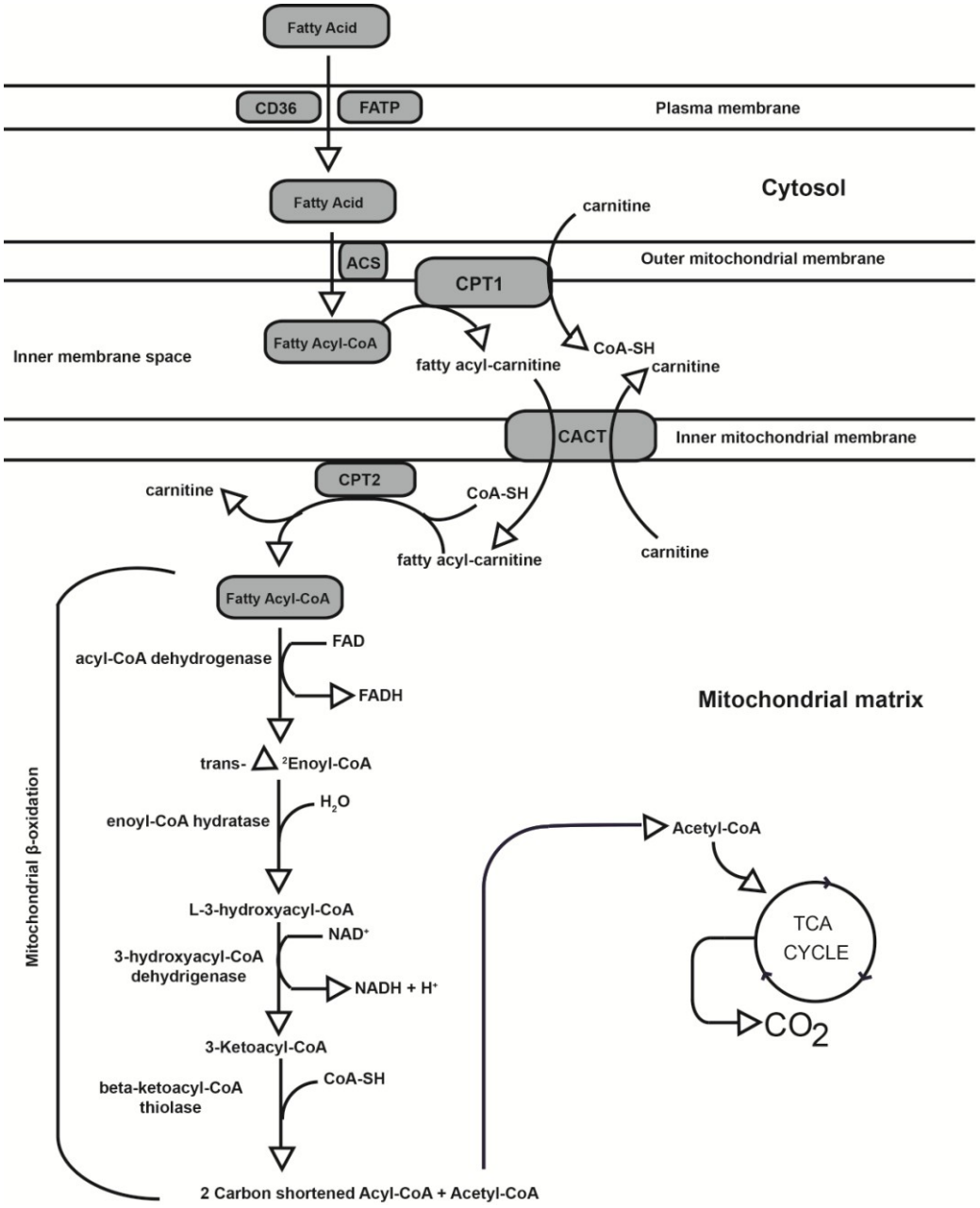


Figure 1-5. **Overview of Fatty Acid Oxidation**. Fatty acids primarily enter a cell via the fatty acid protein transporters, FATP and CD36 on the cell surface. Once inside the cytoplasm, acyl-CoA synthetase (ACS) adds a CoA group to the fatty acid. The fatty acyl-CoA enters the carnitine shuttle system where CPT1 converts the fatty acyl-CoA to a fatty-acyl-carnitine. The fatty acyl-carnitine is transported by the carnitine acyl-carnitine translocase (CACT) across the inner mitochondrial membrane to the mitochondrial matrix. Next, CPT2 converts the fatty acyl-carnitine back to fatty acyl-CoA. The fatty acyl-CoA enters the fatty acid β-oxidation pathway, resulting in the production of one acetyl-CoA from each round of β-oxidation. This acetyl-CoA then enters the TCA cycle. The NADH and FADH2 produced by both β-oxidation and the TCA cycle are used by the electron transport chain to produce ATP.

Metabolism of substrates for energy storage: *De novo* fatty acid synthesis

The pathway for *de novo* fatty acid synthesis uses a different set of enzymes from β-oxidation pathways. *De novo* fatty acid synthesis occurs in the cytoplasm of multiple tissues such as the liver, adipose, brain, and mammary glands. However, the input to fatty acid synthesis begins with cytoplasmic acetyl-CoA, derived mainly from the glycolytic breakdown of glucose. The end product of glycolysis, pyruvate enters the mitochondria and into the TCA cycle where pyruvate dehydrogenase decarboxylates pyruvate to form acetyl-CoA. Next, acetyl-CoA Carboxylase (ACC) catalyzes the biotin-dependent carboxylation of acetyl-CoA to malonyl-CoA in the highly regulated and committed step of fatty acid biosynthesis. ACC is positively regulated by the tricarboxylic acid (TCA) intermediate, citrate, which is also the carbon source for cytoplasmic acetyl-CoA after cleavage by ATP:citrtate lyase. The product of ACC, malonyl-CoA is used by fatty acid synthase (FAS) as the chain elongation unit for the reiterative reductive biosynsthesis of long chain fatty acids. Seven molecules of malonyl-CoA and one molecule of acetyl-CoA interact with FAS in a multi step process to produce the 16 carbon chain length fatty acid, palmitate.

Malonyl-CoA: A Dynamic Metabolic Signaling Molecule

The mechanisms by which obesity leads to insulin resistance and type-2-diabetes have been difficult to parse into one unifying theory. It is clear that insulin resistance is the physiological response of cells to a state of over-nutrition and insulin action. Insulin sensitive, target tissues, such as the liver, can sense changes in nutrition status, termed "nutrient sensing", through a complex network of highly regulated metabolic pathways.

Accordingly, glucose and fatty acids function as the two primary indicators of energy surplus, by serving as substrates for energy sensing pathways. The relationship between lipid and glucose metabolism is intimately connected through coordinated regulation of a small three-carbon metabolite, malonyl-CoA.

Originally identified by Salih Wakil in the late 1950s, malonyl-CoA is produced by the carboxylation of acetyl-CoA by the enzyme, acetyl-CoA carboxylase (ACC) **(Figure 6)**. The reaction requires ATP, biotin, and bicarbonate and occurs in the cytoplasm of mammalian cells **(Figure 7)**. It serves as an intermediate substrate in fatty acid metabolism by serving as the chain elongation unit for fatty acid synthetase (FAS) for the production of long chain fatty acids. Malonyl-CoA also functions as the allosteric inhibitor of carnitine palmitoyltransferase-1 (CPT1), the rate-limiting enzyme in fatty acid oxidation, thereby blocking entry of long chain fatty acids for oxidation in the mitochondria.

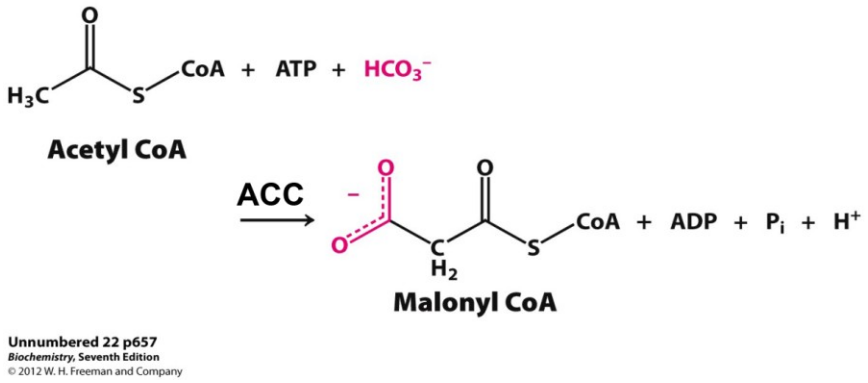


Figure 1-6. Synthesis of Malonyl-CoA. The key regulating enzyme of lipogenesis is acetyl-CoA carboxylase, which catalyzes the synthesis of malonyl-CoA from acetyl-CoA and CO_2. *Modified from source*: *Biochemistry, Seventh Edition. 2012. W. H. Freeman and Company.*

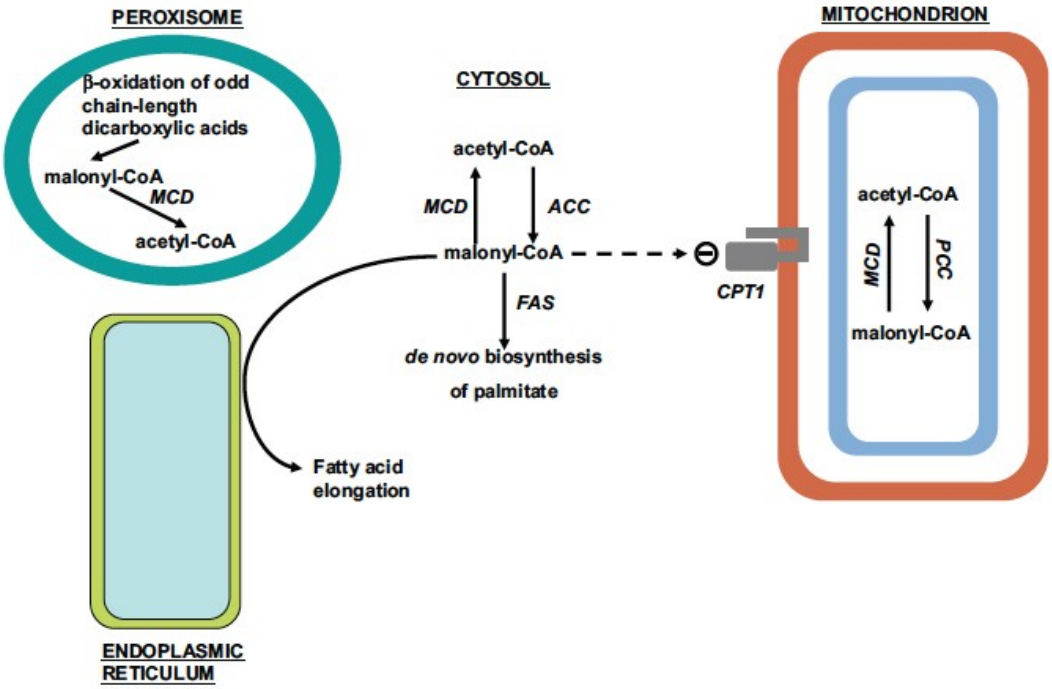


Figure 1-7. Summary of malonyl-CoA metabolism and its compartmentation in mammalian cells. Also shown is carnitine palmitoyltransferase 1 (CPT1), with an indication of its topology in the mitochondrial outer membrane and its inhibition by malonyl-CoA. ACC, acetyl-CoA carboxylase; FAS, fatty acid synthase; MCD, malonyl-CoA decarboxylase; PCC, propionyl-CoA carboxylase. *Saggerson D. 2008, Annu. Rev. Nutr. 28:253-72*

Malonyl-CoA is also formed within the mitochondria and peroxisomes and their physiological significance within these organelles is starting to become clear. Recently, data has emerged to demonstrate malonyl-CoA is actively produced within the mitochondrial matrix. ACSF3 is a mitochondrial malonyl-CoA synthetase enzyme that converts both methylmalonate and malonate into methylmalonyl-CoA and malonyl-CoA, respectively (25). Individuals with mutations in ACSF3 have elevated levels of malonic

and methylmalonic acid in the urine and malonylcarnitine in the plasma, which is thought to cause a range of neurological disorders (26, 27). Although the origin of malonate metabolism in humans is not known, it is a competitive inhibitor of succinate dehydrogenase or Complex II of the electron transport chain (28). Therefore, the function of ACSF3 to is to prevent methylmalonic and malonic acid from accumulating by diverting it to the production of malonyl-CoA. It has been speculated that mitochondrial malonyl-CoA can be used for the production of lipoic acid and the production of long chain fatty acids by the mitochondrial FAS (FAS type II enzyme) (29). However, it remains unknown how changes in mitochondrial malonyl-CoA can affect rates of fatty acid oxidation and synthesis.

ACC and MCD Regulate Malonyl-CoA Concentration

Two opposing enzymes, ACC and MCD, regulate malonyl-CoA concentration in multiple tissues and organelles. ACC is present as two isoforms, ACC1 and ACC2. ACC1 is localized in the cytoplasm and is the predominantly isoform expressed in lipogenic tissues, such as the liver and adipose tissue. ACC1 is thought to function as the provider of cytoplasmic malonyl-CoA for lipogenesis (30, 31). ACC2, positioned on the mitochondrial outer membrane, is found predominately in oxidative tissues, such as the skeletal muscle and heart (32, 33). This isoform is thought to function as the producer of malonyl-CoA near the mitochondrial surface to directly inhibit CPT1 activity and prevent fatty acids from entering oxidative metabolism. Both isoforms are expressed at varying levels in lipogenic and oxidative tissues and they are primarily regulated through allosteric inhibition and protein phosphorylation events. Inactivation of ACC by

phosphorylation is regulated by protein kinase A (PKA) and AMP-activated protein kinase (AMPK) (34, 35). AMPK activation favors fatty acid oxidation by decreasing malonyl-CoA concentration as a result of phosphorylating and inhibiting ACC.

Malonyl-CoA concentration is also dictated by the activity of Malonyl-CoA decarboxylase (MCD), an enzyme whose role is to convert malonyl-CoA to acetyl-CoA and CO_2. Human MCD is encoded by a single gene and produces three isoforms localized to the cytoplasm, mitochondria, and peroxisomes (36). Humans with mutations in the MCD gene display elevated urinary malonic acid, stunted growth, cardiomyopathy, and mental retardation; similar conditions to those seen in individuals with mutation in ACSF3 as described above (37-40). Gene expression studies of human MCD mRNA analysis show skeletal muscle and heart expressing the highest levels of MCD, while the kidney, brain, lung, pancreas contain appreciable levels of activity (36). Its activity and expression has been demonstrated to play a key role in promoting fatty acid oxidation, while pharmacalogical inhibition of the MCD enzyme has been implicated in playing a cardio protective role in ischemia and reperfusion recovery, and displaying toxicity towards human breast cancer cells (41, 42).

Malonyl-CoA Targets CPT1

The role of the mitochondrial carnitine palmitoyltransferase 1 (CPT1) protein is to catalyze the exchange of CoA on a long chain fatty acyl CoA for carnitine. This exchange allows the long chain fatty acyl species to traverse the mitochondrial membranes, with the aid of an additional enzyme, carnitine palmitoyltransferase II (CPT2) and transporter, carnitine-acylcarnitine translocase (CACT), where it can undergo oxidation in the

mitochondrial matrix. In mammalian cells, CPT1 exists as three isoforms that are encoded by three different genes: CPT1A, CPT1B, and CPT1C (43). The liver expresses CPT1A, while the CPT1B isoform is predominately expressed in the heart and skeletal muscle, and brown adipose tissue. The brain expresses all three isoforms, but CPT1C is exclusive to the brain. CPT1C is atypical from the other CPTs because it is located in the ER and does not catalyze the same reaction as CPT1A and CPT1B (44). Metabolomics studies have suggested a role for CPT1C in oxidative stress based on an increase in oxidized glutathione; however, it's precise role remains unclear (45).

CPT1's other physiological role is being a target for malonyl-CoA inhibition during the fed state to prevent the oxidation of long chain fatty acids in the mitochondria (46). The interaction between malonyl-CoA and CPT1 exists in the liver, cardiac and skeletal muscle, brain, and pancreatic β-cells. Even though CPT1A and CPT1B share 62% amino acid identity, the CPT1 isoforms present varying sensitivities toward malonyl-CoA inhibition. CPT1A has a lower affinity for malonyl-CoA than CPT1B, making oxidation more finely tuned in the muscle than in the liver. Works by several labs have demonstrated CPT1A sensitivity is modulated by interaction between its NH_2 and COOH termini and its modulation depends on various physiological states (47).

Metabolic Fuel Switching

Mechanisms have evolved to control the cross talk between pathways of glucose utilization and fatty acid oxidation. The glucose-fatty acid cycle, introduced by Sir Phillip Randle, adds an additional level of control to metabolic fuel switching within tissues on top of the established hormonal regulation by insulin and glucagon (48-50). The cycle

describes the interactions between substrates, in particular, the inhibition of glucose oxidation by fatty acids or ketones without hormonal intervention. During the fasted state, lipolysis is triggered to catabolize stored triacylglycerol (TAG) into glycerol and free fatty acids. Lipoylsis allows free fatty acids to be released into circulation for use by peripheral tissues to meet their energy needs. Oxidation of fatty acids and production of ketone bodies fulfill the local energy needs in the liver while other tissues oxidize ketone bodies. This mechanism allows fatty acids and ketones to spare glucose for the brain during starvation. Inhibition of glucose oxidation occurs at the level of inhibition of hexokinase, phosphofructokinase, and pyruvate dehydrogenase by products generated from the β-oxidation pathway **(Figure 8)**. Randle originally described the mechanism to work in the heart and diaphragm (48). Additional studies have shown the mechanism occurs in the liver, pancreatic β-cells, skeletal muscle and adipose tissue. The glucose fatty acid cycle takes affect in the fed state after a high fat meal or after exercise. Under these circumstances, the non-oxidized glucose is spared and routed toward glycogen production.

In the late 1970's, Dennis McGarry and his colleges demonstrated that high glucose concentrations inhibit fatty acid oxidation through malonyl-CoA mediated inhibition of carnitine palmitoyltransferase-1 (CPT1), the rate-limiting step in fatty acid oxidation. This reciprocal mechanism has been termed the "reverse glucose fatty acid cycle." Changes in malonyl-CoA concentration allow the liver to transition between fed and fasted states. These changes are also regulated by the hormonal stimulation of insulin and glucagon. In the fed state, [low glucagon : high insulin] signals the liver towards fatty acid synthesis by converting glucose to fatty acids and then to formation of long chain

acyl-CoAs. During the fed state, the pool of malonyl-CoA, produced from the ACC reaction, increases and inhibits CPT1, ensuring that the newly formed long chain acyl-CoAs react with glycerol phosphate to form triacgylcerides that are then exported from the liver in the form of VLDL **(Figure 9)**. Conversely, following the shift in the [high glucagon:low insulin] in the fasting or diabetic state, the fatty acid synthesis pathway comes to a halt, the malonyl-CoA concentration falls, CPT1 becomes uninhibited, and fatty acids reaching the liver enter the β-oxidation pathway to form ketone bodies.

The molecular mechanisms regulating these events have increased our understanding of the methods used to exert control over metabolic pathways. Regulation occurs through allosteric control of proteins, reversible phosphorylation, and expression of key enzymes through transcriptional regulation of hormone receptors. Multiple isoforms of glucose transporters (GLUT1-4) exist with differing kinetic properties. The discovery that certain fatty acids can exert transcriptional effects by binding to the nuclear hormone receptor family of peroxisome proliferator-activated receptors (PPARs) adds a new dimension in regulating lipid metabolism by mediating long term effects on substrate metabolism. The PPAR isoforms (PPARα, -β, **-γ)** are differentially expressed amongst multiple tissues to regulate lipid and glucose metabolism.

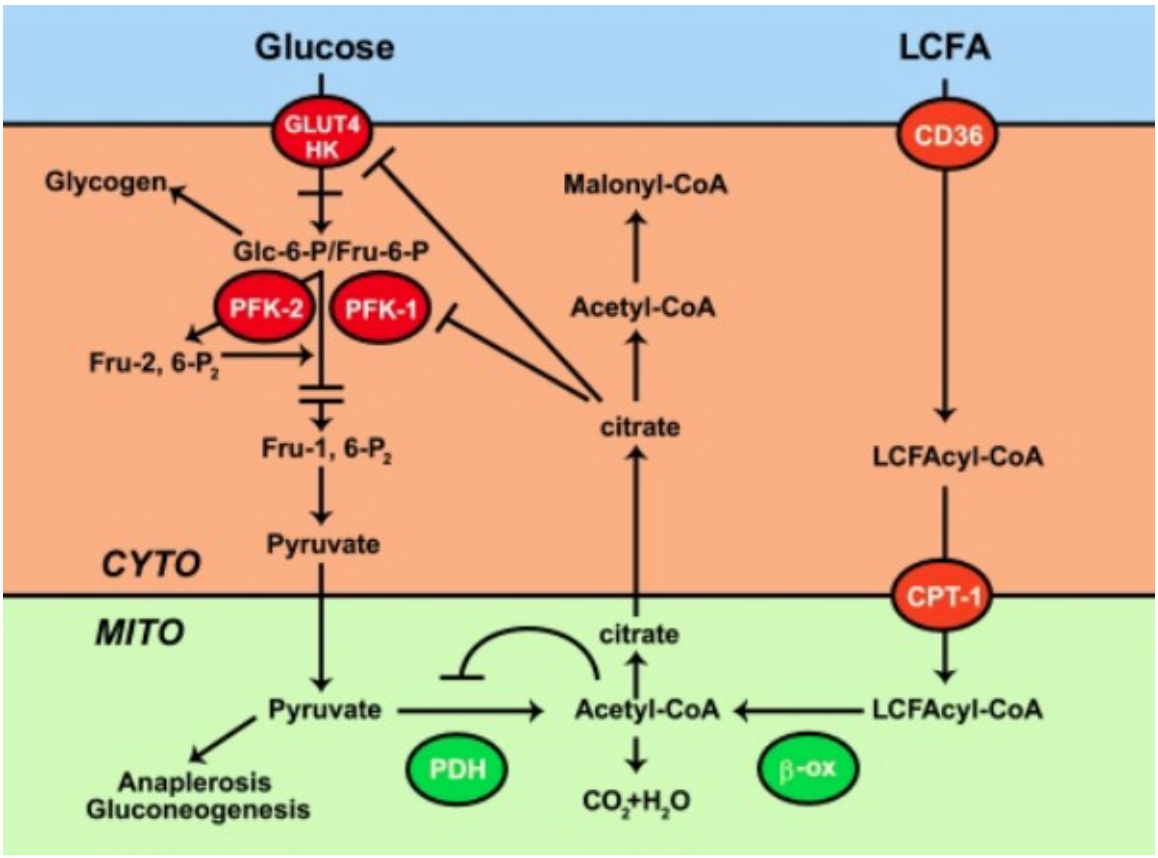


Fig. 1-8. Mechanism of inhibition of glucose utilization by fatty acid oxidation. The extent of inhibition is graded and most severe at the level of pyruvate dehydrogenase (PDH) and less severe at the level of 6-phosphofructo-1-kinase (PFK) and glucose uptake. PDH inhibition is caused by acetyl-CoA and NADH accumulation resulting from fatty acid oxidation, whereas PFK inhbition results from citrate accumulation in the cytosol. The mechanism of inhibition of glucose uptake is not clear. These effects reroute glucose toward glycogen synthesis and pyruvate to anaplerosis and/or gluconeogenesis. CYTO, cytosol; MITO, mitochondria; GLUT4, glucose transporter 4; HK, hexokinase; Glc-6-P, glucose 6-phosphate; Fru-6-P, fructose 6-phosphate; CPT I, carnitine palmitoyltransferase I; β-ox, β-oxidation. *Hue L , and Taegtmeyer H Am J Physiol Endocrinol Metab 2009;297:E578-E591.*

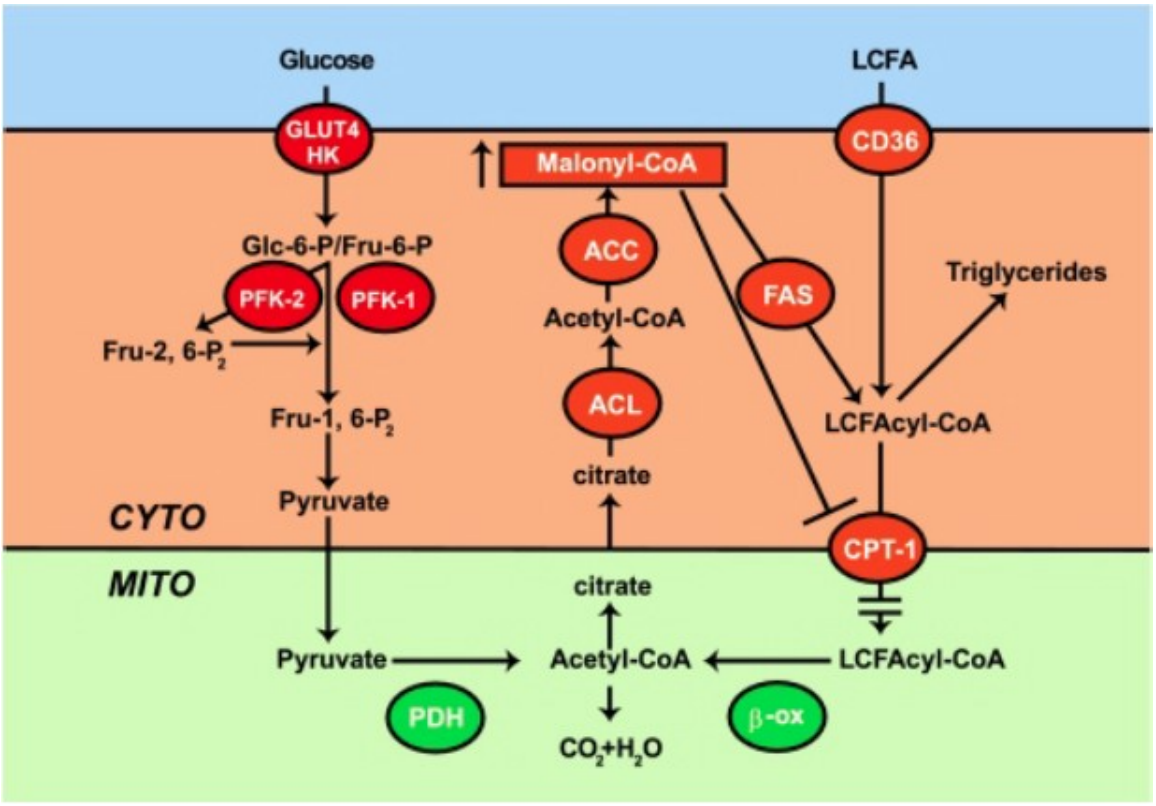


Fig 1-9. Mechanism of inhibition of fatty acid oxidation by glucose. This mechanism is mediated by malonyl-CoA, the concentration of which depends on ACC activity and which inhibits the entry of long-chain fatty acyl (LCFAcyl-CoA) moieties into mitochondria. This effect reroutes fatty acids toward esterification. In extrahepatic tissues, the effect of glucose is stimulated by insulin. ACL, ATP-citrate lyase; FAS, fatty acid synthase. *Hue L , and Taegtmeyer H Am J Physiol Endocrinol Metab 2009;297:E578-E591.*

For example, PPARα is expressed mainly in the liver, kidney, heart, and muscle and stimulates the transcription of genes that are involved in fatty acid uptake and mitochondrial and peroxisomal β-oxidation of fatty acids. PPARα also affects glucose metabolism by increasing PDK4 mRNA and protein to inhibit PDH activity and keeping glucose oxidation levels low in the fasted state.

AMP-activated kinase (AMPK) has also come to be recognized as a mechanism involved in the cell's energy sensing ability. AMPK is expressed in multiple tissues and is activated by metabolic stresses, such as a decrease in glucose supply or oxygen deprivation, or an increase in energy demand, such as exercise. These stresses increase cytosolic concentrations of AMP to rise, altering the [AMP/ATP] ratio. Changes in $[Ca^{2+}]$ can also activate AMPK and involves pathways that change intracellular calcium levels. Exercise stimulates AMPK activation in the skeletal muscle to regulate energy balance by turning on ATP generating pathways (fatty acid oxidation and glycolysis), while switching off ATP consuming pathways (lipid and protein synthesis). AMPK phosphorylates and inactivates its substrate, ACC to lower its enzymatic activity, allowing malonyl-CoA concentration levels to fall by the decarboxylation activity of MCD. This process allows the skeletal muscle to oxidize fatty acids and produce ATP, providing favorable conditions for the muscle to adapt to the exercise induced stress response.

The mechanisms involved in regulating metabolic fuel switching are still evolving and the breadth of contribution to these cycles by enzymes, energy substrates, transcription factors, and genes require a substantial discussion on the topic, that is beyond the scope of this thesis. Overall, metabolic fuel switching involves regulation of glucose, lipid, and protein metabolism to allow tissues to adapt to changes from the environment with the appropriate use of substrates. The inability to promote metabolic fuel switching in insulin responsive tissues has been proposed to be a cause for the rise in insulin resistance. Exactly how these mechanisms work in mediating insulin resistance still remains unclear.

Development of chemical genetic approaches to study metabolism

Our increased understanding of biology stems from the ability to alter biological mechanisms by perturbing the function of genes, proteins and enzymes. This is usually accomplished by the use of traditional pharmacological inhibitors, genetic manipulation of genes in transgenic mice, and RNA interference (RNAi). Each method diverges at levels of complexity, timescale of perturbation, and efficiency. With the development of new methods, researchers are given more flexibility and control in their research design. Chemical genetics is defined as the study of biological systems using small molecule tools. Small molecules are advantageous tools because they can be designed to be cell permeable, selective, function rapidly and reversibly. These approaches provide excellent temporal and quantitative control to study biological processes.

Chemical genetic approaches afford great versatility in the field of metabolism. Traditional approaches used to study metabolic perturbations have involved homologous recombination techniques in mice that enable protein regulation through mutations, transcriptional control through RNAi, or viral mediated gene delivery. These approaches have their limitations. In some cases, genetic attenuation, mutations, and gene knock-outs in mammals are embryonic lethal or have detrimental outcomes during early development that prevent full characterization of animal physiology, particularly at later stages of life. RNAi techniques are problematic because they are temporary and changes in mRNA levels do not always correspond to changes in protein levels. Viral mediated gene delivery methods (adeno-associated virus, lenti virus, retrovirus, etc) are transient, not cell specific and are prone to transfection inefficiency. Because the study of metabolic regulation in whole animal physiology relies on secreted hormone factors, cell-

cell interactions, and multiple isoenzymes, identifying sophisticated methods for controlling the level of any protein with fine tuned precision is necessary. Mediating protein regulation in metabolism with the small molecule technology of chemical genetics, any protein can be targeted for study with tissue and cell specificity, with reversibility of activation.

Overview of Shield-1 Chemical Genetics System

Technology that provides the ability to rapidly and reversibly alter protein function is very desirable to our research investigations. We have made great use of the chemical genetic system developed in the laboratory of Dr. Thomas J. Wandless (Stanford University) based on Shield-1 regulation of a mutant FK506- and rapamycin – bindg protein 12 (FKBP12) to achieve synthetic posttranslational protein stabilization (51). The system utilizes the synthetic chemical ligand, Shield-1 designed to bind to a mutant form of the human FKBP12 protein. Specificity of the Shield-1 ligand to the mutant FKBP12, by conferring selectivity over the non-mutant form of FKBP12, eliminates the problem of off target effects, endemic to traditional pharmacology. The mutant FKBP12 contains two mutations. The first mutation at L106P renders the protein most unstable and targets it for degradation by the proteosome. The second mutation (F36V) allows the binding of Shield-1 to the mutant FKBP12 and stabilizes the mutant FKBP12. Now referred to as a "destabilizing domain," the mutant FKBP12 is fused to a protein of interest (POI). FKBP12 was chosen as a destabilizing domain because its small size, feasibility for developing high affinity ligands, and being a highly studied protein through its binding to the cellular growth and homeostasis regulator, mTOR. Therefore,

the Shield-1 mutant FKBP12 system permits conditional control over protein stability upon addition or removal of the Shield-1 ligand **(Figure 10)**. Fusing the mutant FKBP12 to a fluorescent tag, such as yellow fluorescent protein (YFP), provides a direct visualization of stabilization upon by Shield-1. The system has been shown to be effective in driving conditional and reversible protein control in mammalian cell culture systems, herpes virus, ion channel formation, and mice (51-55).

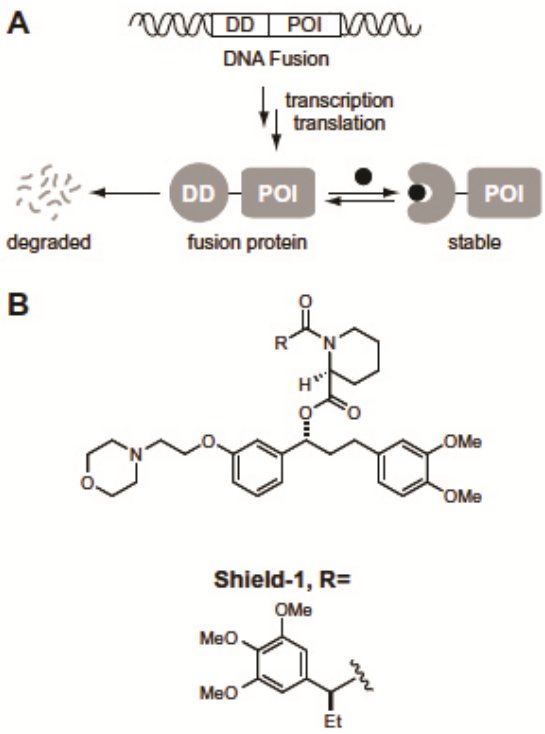


Figure 1-10. A General Method to Conditionally control Protein Stability. (A) Genetic fusion of a destabilizing domain (DD) to a protein of interest (POI) results in degradation of the entire fusion. Addition of a ligand for the destabilizing domain protects the fusion from degradation. (B) Synthetic ligand for FKBP12 F36V, Shield-1. Modified from: *Cell Volume 126, Issue 5 2006 995 – 1004*.

CHEMICAL GENETIC REGULATION OF MALONYL-COA DECARBOXYLASE IN SKELETAL MUSCLE INSULIN RESISTANCE

CHAPTER II: Targeted Chemical-Genetic Regulation of Protein Stability In Vivo*

*Most text and figures in this chapter have been adapted from reference 48

SUMMARY

Loss and gain-of-function transgenic models are powerful tools for understanding gene function *in vivo*, but are limited in their ability to determine relative protein requirements. To determine cell-specific, temporal or dose requirements of complex pathways, new methodology is needed. This is particularly important for deconstructing metabolic pathways that are highly interdependent and cross-regulated. We have combined mouse conditional transgenics and synthetic posttranslational protein stabilization to produce a broadly applicable strategy to regulate protein and pathway function in a cell autonomous manner *in vivo*. Here we show how a targeted chemical-genetic strategy can be used to alter fatty acid metabolism in a recombination and small molecule dependent manner in live behaving transgenic mice. This provides a practical, specific, and reversible means of manipulating metabolic pathways in adult mice to provide novel biological insight.

HIGHLIGHTS

- Simple, broadly applicable method for regulating metabolic proteins and pathways
- Method produces mice that have dually regulated enzyme expression
- Affect function in live mice that is gene specific, reversible, and dose-dependent
- Method requires little knowledge of protein function or enzymatic mechanism

INTRODUCTION

Metabolic pathways are highly interconnected and interdependent. They also exhibit remarkable plasticity that can be easily mistaken for or complemented by redundancy. Understanding these complexities requires specific temporal and reversible pathway perturbation. This is often done using rather nonspecific pharmacology. Ideally, one could use small molecules to selectively and potently alter enzyme activity in an allele-specific manner by means that would require little knowledge of the reaction mechanism. The technique would therefore be straightforward and broadly applicable and would occur posttranslationally.

There is great interest in the development of small molecules that regulate the function of user-engineered proteins (56-60), particularly in the context of intact organisms (61-64). Recently, there has been considerable progress in the development of small stabilizing or destabilizing protein domains that interact with well-defined inert small molecules (51, 52, 65-68). Natural or synthetic small-molecule ligands are chosen that interact with specific proteins to either promote or inhibit their rapid posttranslational degradation (69). Fusion of a protein of interest to these domains creates a small-molecule-regulated protein whose function is dose-dependent and reversible. Here, we have combined ligand-inducible protein stabilization with genetically tractable recombination-mediated transgene expression to enable the targeted small-molecule regulation of enzyme activity in live mice. In order to utilize this strategy to study metabolites in vivo, we developed a transgenic vector that allows the small-molecule regulation of malonyl-CoA decarboxylase (MLYCD) after Cre-mediated recombination.

MLYCD was chosen because its substrate, malonyl-CoA, represents a central metabolic node in fatty acid biochemistry. Malonyl-CoA is the product of the rate-setting step in de novo fatty acid metabolism, is the substrate for fatty acid synthesis and elongation, and allosterically inhibits the rate-setting step in fatty acid beta-oxidation (8). Therefore, exerting control over malonyl-CoA through MLYCD has broad experimental utility. This approach is not limited to MLYCD but is broadly applicable to other enzymes and genes.

RESULTS

Design of a Small-Molecule-Regulated Malonyl-CoA Decarboxylase

In order to produce dose-dependent, small-molecule-regulated posttranslational stabilization, Wandless et al. constructed a modified FK506 binding protein 12 (FKBP12) that binds to and is reversibly stabilized by a synthetic, biologically inert small molecule, Shield-1 (51, 52, 70). We made several modifications to the FKBP12-based destabilization vectors. A picornavirus PTV1-2A peptide-linked mCherry was fused in-frame after an FKBP-eYFP destabilization fusion protein and then cloned into an MMLV-based selectable retroviral vector (Figure 2-1A). The 2A peptide allows two proteins to be produced from a single mRNA at a 1:1 stoichiometric ratio, enabling a ratiometric, autonomous measurement of stabilization and localization within cells (71, 72). Malonyl-CoA decarboxylase (MLYCD) was then cloned as a C-terminal fusion with FKBP-YFP flanked by two short flexible linkers. Fusion of the mutant FKBP12 to YFP linked to MLYCD allows real-time visualization of Shield-1-induced stabilization. The N-terminal mitochondrial localization domain and C-terminal peroxisomal targeting sequences were removed from MLYCD to facilitate cytoplasmic MLYCD activity (73).

Two stable HEK293T stable cell lines were produced that express FKBP12-eYFP-MLYCD-2a-mCherry (FY-MLYCD) or FKBP12-eYFP-2a-mCherry (FYFP) to conduct an initial characterization of this system in vitro. Shield-1 was synthesized as previously reported (51, 74).

In order to verify that the stable cells expressed mCherry constitutively and eYFP in a Shield-1-dependent manner (Figures 2-1A–1D), stable cell lines were treated with Shield-1 for 16 hr. Addition of Shield-1 induced FYFP and FY-MLYCD in a dose-dependent manner (Figures 2-1C and 1D). To determine the kinetics of stabilization, stable cells were incubated with Shield-1 and collected at the designated time points. Saturation of stability was achieved 12 hr posttreatment (Figure 2-1E). Likewise, we conducted a washout experiment to determine the kinetics of destabilization. Stable cells returned to basal stabilization levels 48 hr after Shield-1 removal (Figure 2-1F). These data show that Shield-1 induced MLYCD stabilization in a dose-dependent and reversible manner.

To demonstrate that the FY-MLYCD fusion protein was enzymatically active, we treated FY-MLYCD stable cells with Shield-1 or vehicle control for 16 hr. Indeed, Shield-1 induced an ~4-fold increase in MLYCD activity (Figure 2-2A). In a second experiment, increasing amounts of Shield-1 were added to FY-MLYCD cells and then the cells were assayed for ^{3}H-acetate incorporation into lipids, a measure of de novo fatty acid synthesis. Addition of Shield-1 resulted in a dose-dependent suppression of de novo fatty acid synthesis, demonstrating that FY-MLYCD was enzymatically active and its activity was regulated by Shield-1 in a dose-dependent manner (Figure 2-2B). These

experiments show that the fusion of the mutant FKBP12 to MLYCD maintained an active enzyme that can be regulated by Shield-1.

Dual Chemical and Genetic Regulation of Fatty Acid Metabolism In Vivo

Shield-1 has been shown previously to be active when injected into mice, either intravenously or intraperitoneally, in a tumor xenograft model to regulate secreted fusion proteins in vivo (52). Given the success of Shield-1 in mice, we proceeded to modify this technology into a genetically tractable and targeted in vivo mammalian expression system to affect metabolism in live behaving mice. The vector was designed to enable the conditional targeted expression of a Shield-1-regulated cassette in transgenic mice. A lox2272-flanked eCFPcaax stop cassette was cloned into a ubiquitous mammalian expression vector (75). Lox2272 was chosen because it does not recombine with traditional loxP sequences used in most conditional knockout (KO) mice (76). The FY-MLYCD transgene was cloned downstream of the floxed stop cassette (Figure 2-3A). FY-MLYCD is produced only after Cre-mediated excision of the eCFP stop cassette. Transgenic mice expressing Lox2272-eCFPcaax pA Lox2272-FKBP12-eYFP-MLYCD-2a-mCherry (Tg-fMCD) were produced and characterized for eCFP expression by Western blot and direct epifluorescent visualization in cryosections. CFP expression was the highest in the muscle, pancreas, and kidney with little to no expression in the brain and liver (Figure 2-3B). These mice were then crossed to mice that ubiquitously express a tamoxifen-inducible Cre recombinase, CRE^{ERT2} (77) to produce double-transgenic mice ($Tg\text{-}fMCD^{ERT2}$) in order to initially characterize the Shield-1-inducible system in multiple tissues. These Shield-1-regulated transgenic mice can be bred to any transgenic Cre-

recombinase-expressing mouse to achieve tissue specificity or temporal regulation with inducible Cre mice, or both. The flexibility of having dual inducible systems is particularly beneficial in cases where the induction of genes is desired in both a cell-specific and temporal manner. This transgenic vector can be easily tailored to meet individual needs.

Primary mouse embryonic fibroblasts (MEFs) were derived from double-transgenic mice. The MEFs exhibited membranous eCFP expression as a marker for transgene expression. The addition of 4-hydroxy-tamoxifen resulted in recombination and expression of mCherry (marker for recombination), but not of FY-MLYCD (Figures 2-3C and 3F). Addition of Shield-1 resulted in the expression of cytoplasmic FY-MLYCD (Figure 2-3F) in a dose-dependent manner (Figure 2-3C). We conducted kinetic studies to determine the stabilization of FY-MLYCD after 4-hydroxy-tamoxifen and Shield-1 addition. Peak stabilization was reached at 24 hr (Figure 2-3D), while washout studies showed that MEFs returned to basal stabilization levels 96 hr after Shield-1 removal (Figure 2-3E). These results demonstrate that MLYCD is dually regulated by Cre-mediated recombination and Shield-1 ex vivo.

Next, we tested this dual regulatory system in vivo. Tg-fMCD^{ERT2} animals were injected four times with 200 mg/kg intraperitoneal (i.p.) tamoxifen over 1 week to induce recombination of the eCFPcaax domain in vivo. After a 48 hr rest period, mice received three i.p. injections of Shield-1 at 10 mg/kg at 12 hr intervals. Transgene expression was assessed by the expression of membranous eCFP (Figure 2-4A). Recombination was seen in vivo as the expression of untargeted mCherry. Stabilization was visualized by the expression of cytoplasmic eYFP. Little to no expression of eYFP can be seen in the

absence of Shield-1 (Figure 2-4A). Destabilization occurs robustly in vivo, as almost no expression was seen in the absence of Shield-1. mCherry and eYFP expression fully overlapped. No cells were observed expressing eYFP in the absence of mCherry expression. Shield-1 stabilized FY-MLYCD in tissues that express the transgene, including the pancreas, skeletal muscle, heart, and adipose tissue (Figures 2-4B–4E). The Tg-fMCD^{ERT2} mice exhibited dual regulation of FY-MLYCD in multiple tissues.

Finally, we tested the ability of Tg-fMCD^{ERT2} mice to alter the oxidation of fatty acids in live mice. Two genotypes were chosen, Tg-fMCD^{ERT2} and control Tg-fMCD mice. Tamoxifen was administered to all mice as described above to mediate recombination and expression of the FYMLYCD transgene in Tg-fMCD^{ERT2} mice. Mice were then given either Shield-1 or vehicle as above. The mice were then assayed individually for their ability to fully oxidize radiolabeled palmitate to CO_2. Shield-1 had no effect on control mice, however, Shield-1 induced an ~3-fold increase in fatty acid oxidation in the fed state (Figure 2- 5). This is approximately the increase in fatty acid oxidation seen during prolonged fasting (44). Here, we were able to achieve robust changes in the metabolism of fatty acids in live mice using an inert small molecule while simultaneously using wild-type littermates to control for any potential off-target effects of the chemical. These experiments provide the proof of principle for a broadly applicable means to alter protein and metabolic function in vivo.

Tissue-Specific and Dose-Dependent Small-Molecule Regulation of Malonyl-CoA Decarboxylase In Vivo

The ubiquitous tamoxifen-inducible Cre-recombinase-expressing mouse used

above has the advantage of temporal (chemical) and ubiquitous control of recombination. These mice were useful for monitoring basic pharmacodynamics of stabilization in multiple tissues, as well as permitting mosaic recombination to serve as an additional internal cell-autonomous control (Figure 2-4A). This approach is not ideal, however, for dissecting tissue-specific effects of metabolic pathways in the context of whole-animal physiology. To test this system in a tissue-specific context, we crossed our transgenic mice (Tg-fMCD) to mice expressing Cre recombinase from the human alpha-skeletal muscle actin promoter (78) (Tg-fMCD^{skel}). The expression of the muscle-specific Cre recombinase resulted in the deletion of eCFPcaax in muscle without deletion in nonparenchymal tissue, as expected (Figure 2-6A). This strategy resulted in a more uniform expression of the transgene as assessed by mCherry expression. Again, the administration of Shield-1 to Tg-fMCD^{skel} mice resulted in the induction of eYFP-MLYCD that increased with an increasing dose of Shield-1 (Figure 2-6B). These experiments show the utility of this method for modifying protein stability in a tissue-specific and dose-dependent manner in live mice.

DISCUSSION

Here, we have combined inducible protein stabilization with Cre-mediated conditionally targeted mouse transgenics to produce a broadly applicable and straightforward chemical-genetic strategy to affect metabolism in vivo. Chemical biology and chemical genetic techniques have been developed to probe the function of proteins in situ in a protein-/gene-specific manner (56-64). The use of designer small molecules

that can acutely but specifically control the function of targeted, but not native, proteins have advantages in basic science over classical pharmaceutical approaches. The specificity of small molecules for their targets is inversely proportional to their popularity. In other words, all small molecules have off-target effects that are sometimes not appreciated decades after their introduction. In the clinic this can be advantageous or catastrophic (79). In basic science, these effects can be misleading in the absence of target-specific control experiments in knockout mice, which are rarely feasible. Designer drug-target interactions while not useful in the clinic, can be more exquisitely controlled in a laboratory setting.

The transgenic approach we have designed contains several built-in controls to allow direct visualization of stabilization, recombination, and cellular localization simultaneously. The addition of lox2272 sites makes this approach ideal for adding back wild-type or mutant proteins that have been conditionally knocked out using the more common loxP recombination sites. Lox2272, while still being recognized by Cre recombinase, does not efficiently recombine with LoxP, thus mitigating genomic rearrangement. Ideally one could temporally add back a protein into the same tissue it was deleted from and control its function via Shield-1-induced stabilization or destabilization. This approach would be ideal for lethal null alleles or highly compensated pathways. Furthermore, other orthogonal methods can be used in conjunction with this system to achieve multiple modes of regulation in vivo (66, 68, 80).

This simple recombination-based system has the advantage of rapid development, cost effectiveness, and versatility compared to more laborious knockin strategies. The production of mice expressing transgenes is relatively straightforward and accessible to

most researchers. Transgenesis combined with recombination-mediated transgene activation additionally takes advantage of the large number of publically available tissue-specific CRE-expressing mouse lines. Therefore, one can target gain-of-function or gene replacement to specific cell types to better dissect tissue-specific roles of ubiquitous genes. Making knockin mice enables the endogenous expression of genes but does not make it possible to discern tissue-specific effects. Tissue-specific promoters would satisfy this as well but are not versatile in directing transgenes to different cell types. The methodology here is beneficial in cases where one may need to produce several groups of mice to examine, for example, the relative role of enzymes in multiple tissues.

Low-abundance, structurally similar metabolites can be onerous to functionally elucidate within the context of mouse physiology, particularly when defining cause-and-effect relationships. The technique described here has the potential to intimately dissect metabolic pathways in living mice to better understand the relationship between metabolites and metabolic pathways and disease in distinct cell types. For example, acetyl-CoA carboxylases alpha (ACACA) and beta (ACACB) have been assigned differential roles in directing fatty acid metabolism, but these roles have become unclear of late (81-84), presumably due to compensation by these closely related enzymes. Altering specific metabolites in a cell-specific and temporal manner will offer a more precise and nuanced understanding of how these enzymes and pathways contribute to metabolic phenotypes.

SIGNIFICANCE

This mode of posttranslational control over protein stability has the advantage of quick kinetics, dose responsiveness, and reversibility similar to that of small-molecule pharmacology, with the added benefit of allele specificity. This simple, single-vector approach to making double-conditional transgenic mice provides a flexible platform to more precisely refine protein function in vivo. Furthermore, the reaction mechanism does not need to be known, and large-scale chemical screening does not need to be preformed. Therefore, new proteins or structurally similar protein families can be targeted to understand their function in mouse physiology in vivo.

ACKNOWLEDGEMENTS

We thank T.L. Wandless (Stanford University) and E. Provost (Johns Hopkins University) for critical reagents. This work was supported in part by the American Heart Association (SDG2310008 to M.J.W.) and NIH NINDS (NS072241 to M.J.W.).

EXPERIMENTAL PROCEDURES

Constructs

The FKBP12 destabilizing domain construct was fused to eYFP and a murine malonyl-CoA decarboxylase cDNA. Several modifications were made to the MLYCD cDNA prior to fusion with FKBP12-YFP: the mitochondrial and peroxisomal targeting signals were removed with the addition of flexible GSG linkers to the N and C termini of MLYCD. Next, a viral 2A bicistronic peptide was fused in frame linked to the red fluorescent protein mCherry to follow cellular localization. The construct and control (lacking MLYCD) were cloned into the retroviral vector, pLPCx (Clonetech), by standard methods.

Generation of HEK293 Stable Cell Lines Expressing FKBP12-YFP-MLYCD-2A-mCherry

HEK293T cells were grown in DMEM containing 10% FBS and 1% pen/strep (Invitrogen) at 37°C in a humidity-controlled chamber at 5% CO_2. To produce stable cell lines, HEK293T cells were infected with the retroviral constructs. Stable cell lines were produced by antibiotic selection with puromycin.

Generation of Transgenic Mice

A lox2272-flanked eCFP caax STOP SV40 polyA cassette was produced by PCR using standard methods and cloned into the pCAG vector (addgene #13787) (75, 85). The FKBP12-YFP-MLYCD-2A-mCherry transgene was cloned downstream of a Lox 2272 CFP STOP Lox2272 cassette driven by the ubiquitous CMV-enhanced chicken beta actin promoter (CAG). The transgenic vector was injected into pronuclei of fertilized zygotes

(B6SJLF1) at the Johns Hopkins University School of Medicine Transgenic Mouse Core Facility. Transgenic founders and germline transmitters were identified by testing for the expression of CFP by PCR genotyping. We crossed the resulting Tg-fMCD transgenic mouse to a transgenic line that expresses a CRE^{ERT2} inducible by tamoxifen (B6;129S-Tg(UBC-cre/ERT2)1Ejb/J) (77) to study the temporal regulation of MLYCD expression (referred to as Tg-$fMCD^{ERT2}$) or to mice expressing Cre from a muscle-specific promoter (B6.Cg-Tg(ACTA1-cre)79Jme/J) (78) (referred to as Tg-$fMCD^{skel}$). Cre-mediated excision of eCFP is confirmed by genotyping PCR. All procedures were performed in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals and under the approval of the Johns Hopkins Medical School Animal Care and Use Committee.

Assay for De Novo Fatty Acid Synthesis

Stably transfected HEK293T cells expressing FY-MLYCD were plated at a density of 4×10^5 cells in a 24 well plate (n = 6) and stimulated with Shield-1 or vehicle at increasing concentrations for 18 hr. Cells are labeled with 1.0 μCi of [^{3}H] acetic acid acetate (Perkin Elmer) for an additional 2 hr. Total lipids are extracted with chloroform/methanol and counted via liquid scintillation.

Malonyl-CoA Decarboxylase Assay

A fluorimetric assay that measures the formation of acetyl-CoA from malonyl-CoA, coupled with citrate synthase and malate dehydrogenase, was used to determine MCD activity in stable HEK293 T cells expressing FY-MLYCD (86). Cells were plated at a density of 1×10^6 cells in a 10 cm plate, then stimulated with Shield-1 or vehicle for

24 hr. Cells were harvested with an extraction buffer (0.1 M Tris, pH 7.4, containing 1 mM dithiothreitol (DTT), protease, and PhosStop inhibitors cocktail (Roche)) followed by a brief sonication. Cells were pelleted by centrifugation at 13,000 × rpm (30 min at 4°C) and supernatants were transferred to new Eppendorf tubes. Protein estimation was determined using the Pierce BCA Protein Assay Kit, and 0.5 mg of protein was used to carry out a partial purification of MCD by $(NH_4)_2SO_4$. To the lysates, 3.8 M $(NH_4)_2SO_4$ was slowly added on ice with vortexing, until 60% saturation was reached. The mixture was allowed to precipitate overnight at 4°C followed by centrifugation at 13,000 × rpm (60 min at 4°C). The pellets were resuspended in 0.1 M Tris-HCl (pH 8.0). MCD activity was measured using a Synergy MX multimode microplate reader (BioTek Instruments, Inc.) with excitation and emission wavelengths set to 340 and 460, respectively. Reaction mixtures of 140 µl containing 0.1 M Tris-HCl (pH 8.0), 0.5 mM DTT, 10 mM L-malate, 0.5 mM NAD, and 17 units of malate dehydrogenase were incubated for 7 min at 37°C in a 96 well black flat-bottom assay plate. A baseline-fluorescence measurement was followed by the addition of citrate synthase (3.14 units), succeeded by an additional 2 min incubation. Malonyl-CoA (0.3 mM) was added, followed by 20 µl of the $(NH_4)_2SO_4$-purified sample fraction. The incubation was allowed to continue for an additional 7 min before a second measurement for the rate of formation of NADH was taken.

In Vivo Fatty Acid Oxidation

In vivo oxidation of 1-^{14}C palmitic acid was measured in mice by i.p. injection of labeled palmitate (1 µCi) bound to fatty-acid-free bovine serum albumin, as previously reported (44, 75). Mice were individually housed in metabolic chambers and expired $^{14}CO_2$ was

collected by bubbling the collected gas through 1 N NaOH. An aliquant of NaOH was measured by scintillation counting every 15 min for 45 min.

Western Blots

Whole tissues or stable HEK293T cells were harvested and total protein was extracted with sonication in extraction buffer (1% (v/v) Nonidet P-40 in TBS (50 mm Tris-HCl, pH 7.5, 150 mm NaCl) with protease and PhosSTOP phosphatase inhibitors cocktail (Roche). Cellular debris was pelleted at 13,000 × rpm (30 min at 4°C). Protein concentration was estimated using the Pierce BCA Protein Assay Kit. Soluble proteins (25 or 50 μg) were separated using Bio-Rad Tris Glycine SDS-PAGE gels (8%) or Invitrogen BisTris SDS-PAGE gels (4%–12%). Proteins were transferred to nitrocellulose membranes, blocked in 3% nonfat milk and detected by immunoblotting with antibodies to FKBP12 (Thermo Scientific), dsRED (Clonetech), MLYCD (abcam), and Heat shock Protein 8 (HSC-70) (Santa Cruz Biotechnology). HRP was detected using Immobilon Western Chemiluminescent substrate (Millipore Corporation, Billerica, MA) and the FlourChem Western blot imaging system (Cell Biosciences).

FIGURES

Figure 2-1 Small molecule induced enzyme stabilization. (**A**) An FKBP12 destabilization domain that is inducibly stabilized by the small molecule Shield-1 was fused to eYFP and Malonyl-CoA Decarboxylase (MLYCD). The inducible MLYCE is linked to a constitutively expressed mCherry via a bicistronic viral PTV1-2A peptide. (**B**) Epifluorescent images of Shield-1 stabilized MLYCD and constitutive expression of mCherry in HEK 293T cells. Western blots of dose-dependent Shield-1 induced stabilization of (**C**) control and (**D**) MLYCD HEK 293T cells. (**E**) Time course and (**F**) washout of stabilization in HEK 293T cells.

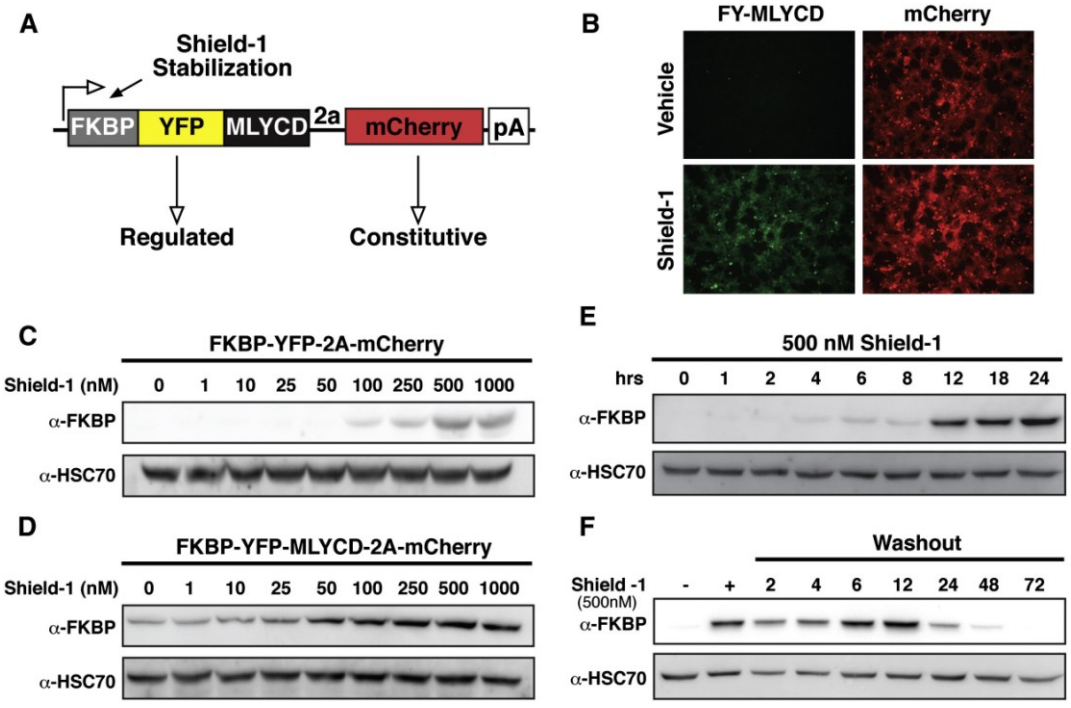


Figure 2-2. Shield-1 regulated Malonyl-CoA Decarboxylase activity. (A) Shield-1 induces MLYCD activity. (B) Shield-1 suppresses *de novo* fatty acid synthesis in a dose-dependent manner as measured by ^{3}H-acetate incorporation into lipids.

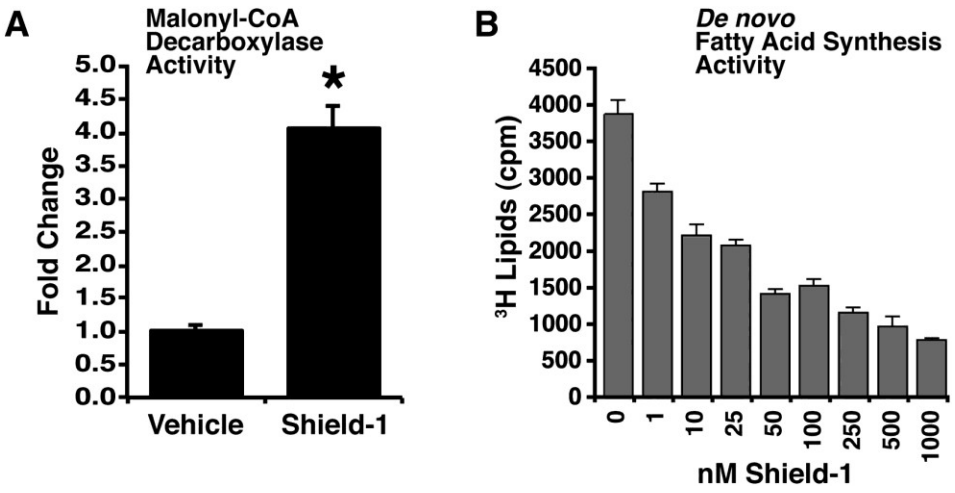


Figure 2-3. Dual chemical regulation of protein stability. (A) The destabilizationcassette was cloned downstream of a Lox2272 eCFPcaax Stop cassette driven by the ubiquitous CMV-enhanced chicken beta actin promoter (CAG). Cre-mediatedrecombination between the Lox sites excises the eCFPcaax Stop cassette and express the FKBP12-YFP-MLYCD-2A-mCherry cassette. (B) Differential eCFP tissue expression in transgenic mice as shown by Western blot. (C) Western blot of MEFs from double conditional Tg-fMCD$_{ERT2}$ mice. Isolated MEFs were treated with 500nM 4-hydroxytamoxifen (4HT) and Shield-1 or vehicle control and blotted for FKBP12 (stabilization), mCherry (recombination), and HSC 70 (loading control). (D) Time course and (E) washout stabilization of MLYCD. (F) Epifluorescent images of tamoxifen inducible Shield-1 stabilized primary MEFs.

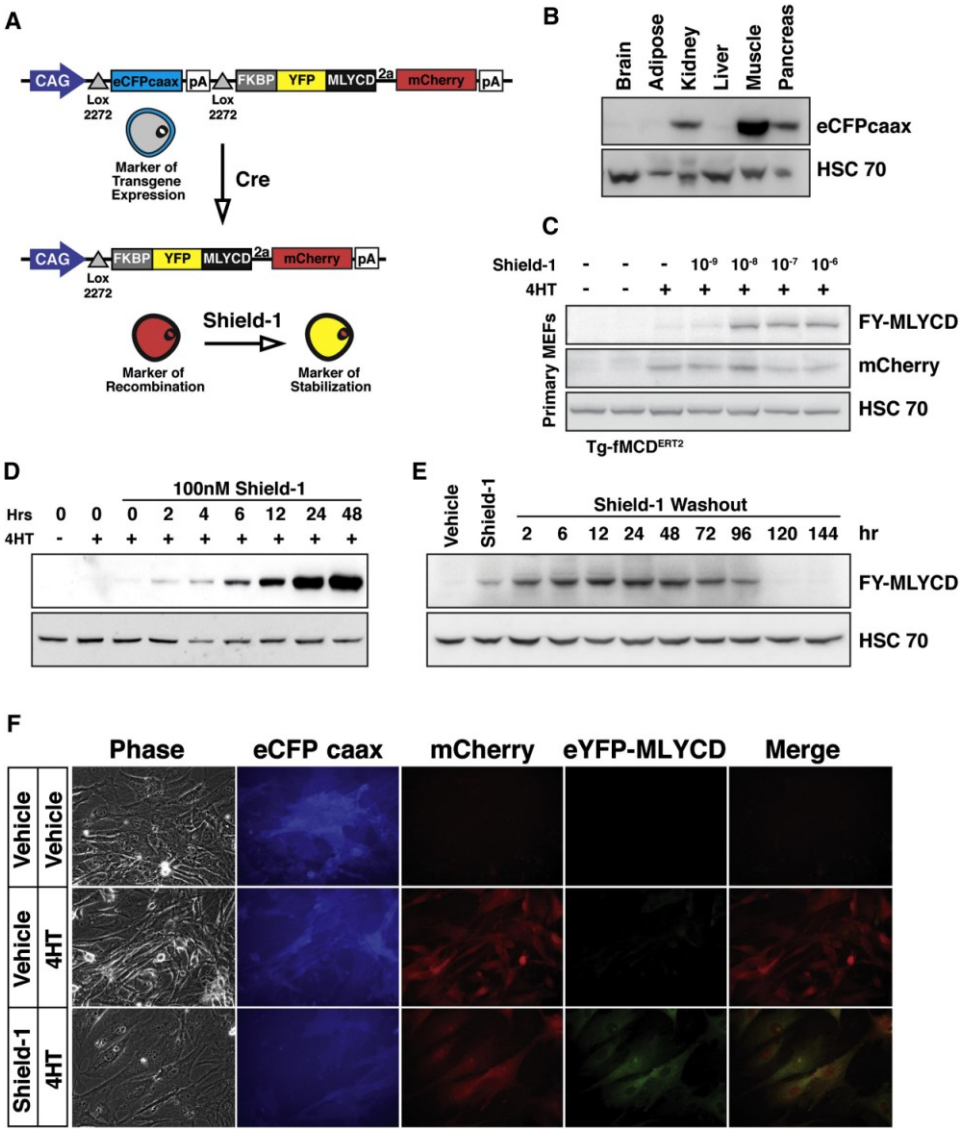


Figure 2-4. Figure 2-4. Chemical genetic regulation of MLYCD *in vivo*. (A) Fluorescent confocal images of Shield-1 stabilization in the pancreas of conditionally regulated Tg-fMCD^{ERT2} mice. Animals were injected with tamoxifen (200 □g/kg), followed by Shield-1 (10mg/kg) or vehicle control. (B) Western blots of FY-MLYCD stabilization by Shield-1 in he pancreas, skeletal muscle, heart, and adipose tissue of Tg-fMCD^{ERT2} mice.

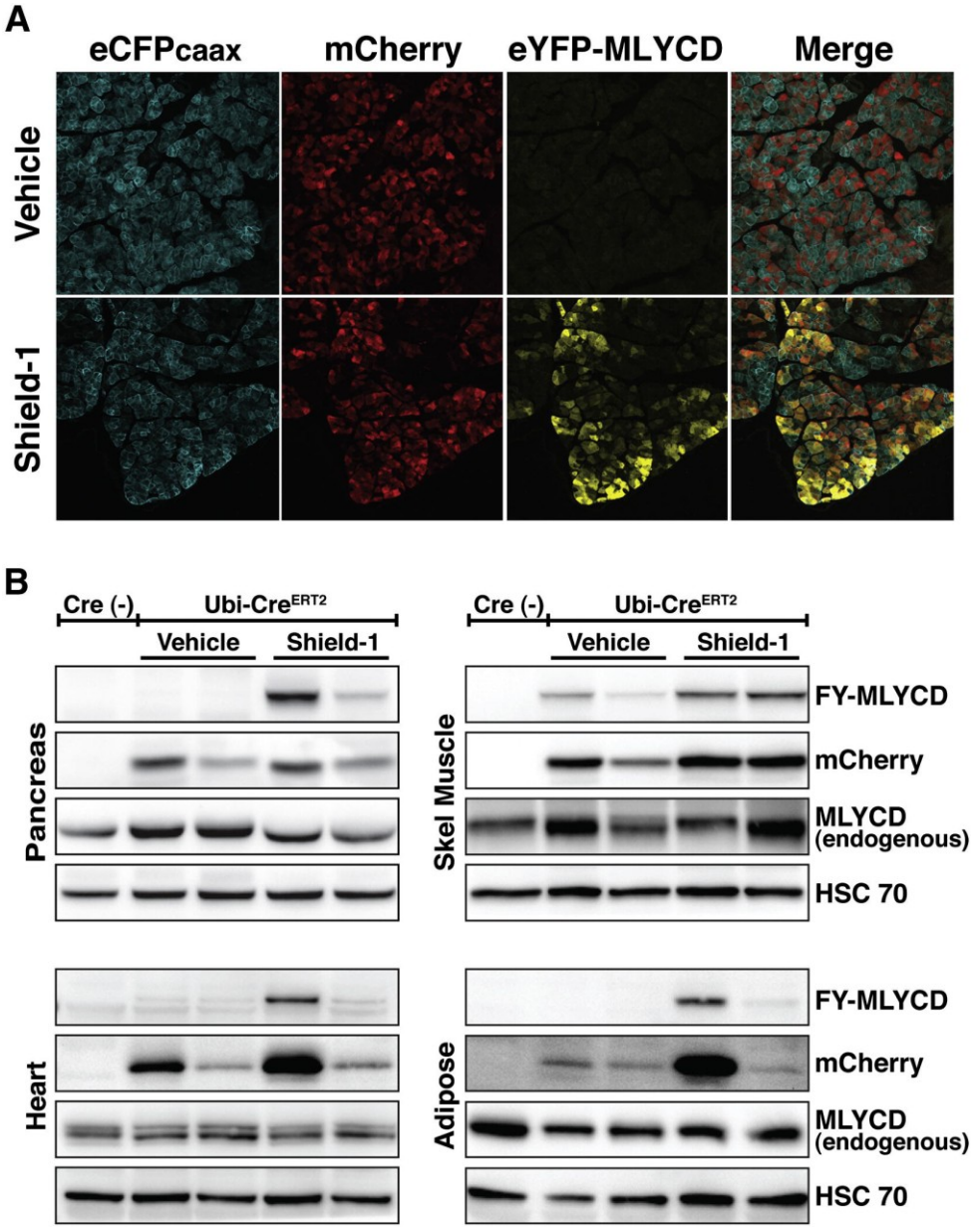


Figure 2-5. Chemical genetic regulation of fatty acid metabolism *in vivo*. Live mouse in vivo oxidation of 1-^{14}C labeled palmitic acid to $^{14}CO_2$ for 45 min. n=3/group, *p<0.01.

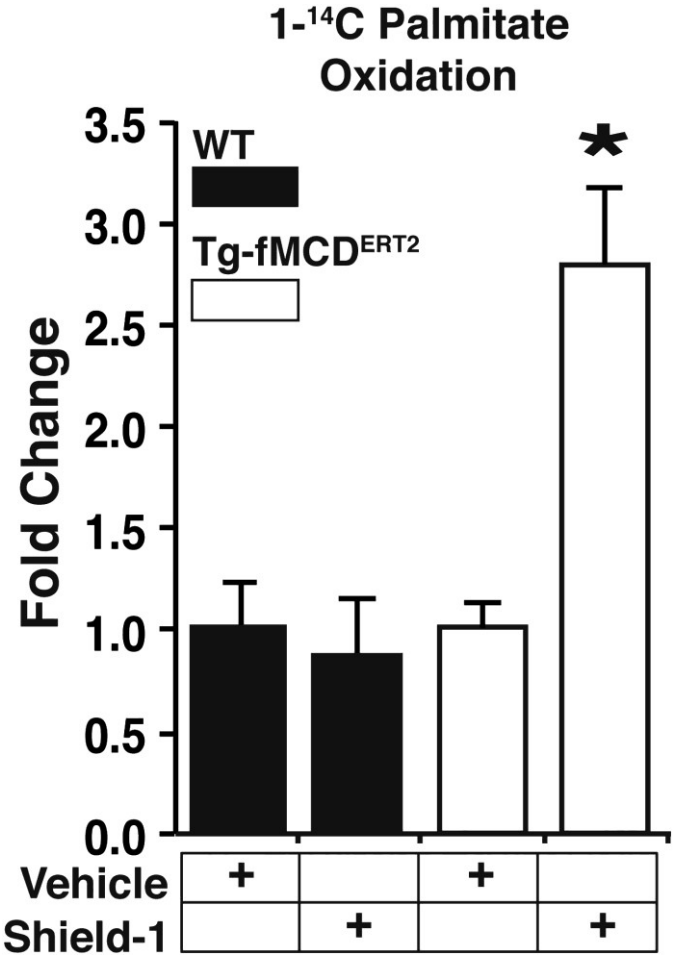
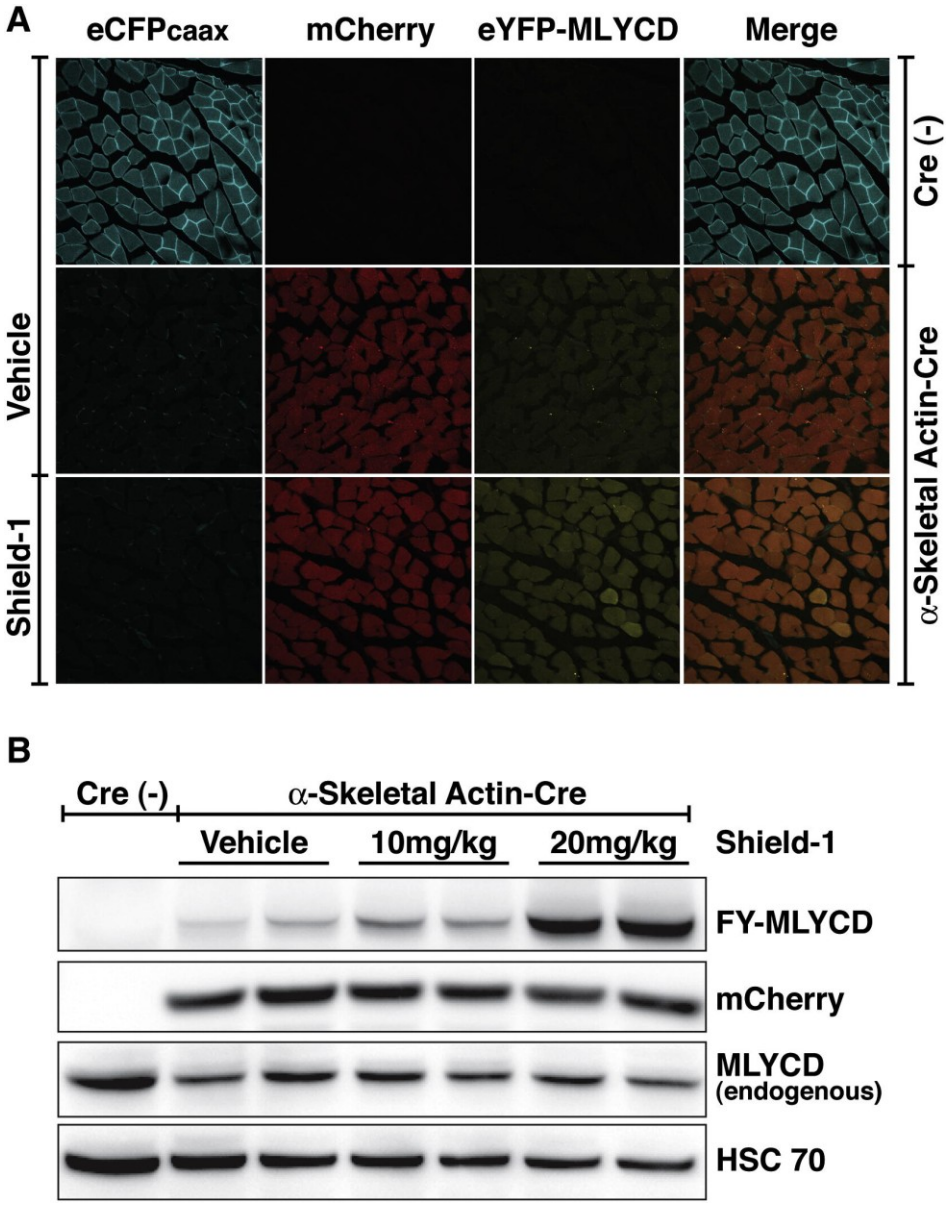


Figure 2-6. Tissue-specific and dose-dependent regulation of protein stability *in vivo*. (A) Images of Shield-1 stabilization in the skeletal muscle of conditionally regulated TgfMCDskel mice by fluorescent confocal microscopy. Animals were injected with Shield-1 (20 mg/kg) or vehicle control. (B) Western blot of Shield-1 dose-dependent stabilization in the skeletal muscle of Tg-fMCDskel and control mice. Animals were injected with Shield-1 (10 mg/kg or 20mg/kg) or vehicle control.



CHEMICAL GENETIC REGULATION OF MALONYL-COA DECARBOXYLASE IN SKELETAL MUSCLE INSULIN RESISTANCE

CHAPTER III: In vivo Chemical-Genetic Induction of Skeletal muscle Malonyl-CoA Decarboxylase Does Not Improve Insulin Sensitivity

SUMMARY

Defects in skeletal muscle fatty acid oxidation have been implicated in the etiology of insulin resistance. Malonyl-CoA decarboxylase (MCD) has been a target of investigation because it reduces the concentration of malonyl-CoA, a metabolite that inhibits fatty acid oxidation. The *in vivo* role of muscle MCD expression in the development of insulin resistance remains unclear. To determine the role of MCD in skeletal muscle of diet induced obese and insulin resistant mouse models we generated mice expressing a muscle specific transgene for MCD (Tg fMCD^{Skel}) stabilized posttranslationally by the small molecule, Shield-1. Tg fMCD^{Skel} and control mice were placed on either a high fat or low fat diet for 3.5 months. Obese and glucose intolerant as well as lean control Tg fMCD^{Skel} and nontransgenic control mice were treated with Shield-1 and changes in their body weight and insulin sensitivity upon induction of MCD were determined. Two weeks of MCD induction did not improve body weight or glycemic control in obese Tg fMCD^{Skel} mice. In obese Tg fMCD^{Skel} mice, skeletal muscle insulin-stimulated AKT phosphorylation was decreased compared to obese nontransgenic controls. In addition, skeletal muscle specific expression of MCD decreases the protein and mRNA abundance of oxidative metabolism genes compared to obese controls. Acute induction of skeletal muscle MCD in obese and glucose intolerant mice did not improve body weight and decreased insulin sensitivity compared to obese nontransgenic controls. However, induction of MCD in skeletal muscle resulted in a suppression of mitochondrial oxidative genes suggesting an unexpected redundant and metabolite driven regulation of gene expression.

INTRODUCTION

The concomitant rise in obesity and type 2 diabetes has mustered a global effort to understand the links between nutrient overload and insulin resistance to enable new therapies. The skeletal muscle plays an important role in maintaining systemic glycemic control by mediating a majority of insulin stimulated glucose disposal. Skeletal muscle has been demonstrated to be a primary tissue driving insulin resistance and is the target for several anti-diabetic drugs (87-89). Excess lipid accumulation outside of adipose tissue is thought to contribute to diabetes by engaging pathways that inhibit insulin signaling. The mechanisms that lead to the development of lipid induced insulin resistance remain elusive, but consistent themes converge at pathways implicating inflammation, ER stress, and mitochondrial insufficiency (12, 15, 16, 90).

Skeletal muscle with its high capacity for fatty acid oxidation has been a focal point for genetic and pharmacological studies aimed to restore lipid balance by promoting lipid oxidative pathways. Mitochondria are the major site for fatty acid oxidation and defects in this process may contribute lipotoxic pathways. The lipotoxicity hypothesis suggests that accumulation of lipid signaling intermediates interact and disrupt insulin signaling to mediate or exacerbate insulin resistance (91-95). The finding that the muscles of patients with type 2 diabetes contained fewer and smaller mitochondria than those of age matched insulin sensitive controls, further supported the concept that mitochondrial deficiency or dysfunction is a driver of insulin resistance (96-102). The muscle's decreased mitochondrial content limits its capacity to oxidize fatty acids, resulting in the accumulation of undesirable intramuscular lipids, such as ceramide, TAG, and DAG. Therefore, methods that increase fatty acid oxidation akin to exercise, in the

muscle to relieve the toxicity caused by these lipid intermediates have been sought to improve insulin resistance.

Malonyl-CoA is the substrate for de novo fatty acid synthesis and its concentration is dependent on the nutritional status of the cell. Malonyl-CoA is produced by acetyl CoA carboxylase (ACC) and catabolized by malonyl-CoA decarboxylase (MLYCD, commonly referred to as MCD) in the cytoplasm. Although malonyl-CoA is the substrate for fatty acid synthase (FAS) for the production of fatty acids de novo, it does not occur at high levels in skeletal muscle (103). In the skeletal muscle, MCD catalyzes the decarboxylation of malonyl-CoA to enhance fatty acid oxidation as malonyl-CoA is the allosteric inhibitor of the rate setting step in mitochondrial long chain fatty acid beta-oxidation, Carnitine Palmitoyltransferase 1 (CPT1). Genetic and pharmacological studies to inhibit or activate genes involved in fatty acid synthesis (ACC2) or oxidation (CPT1, MCD, AMPK) have produced conflicting results into the benefits of skeletal muscle mitochondrial fatty acid oxidation in models of diet induced insulin resistance (73, 81, 83, 104-107). A whole body deletion of ACC2, to promote fat oxidation by decreasing malonyl-CoA levels, produces lean hyperphagic mice. As a result, $ACC2^{-/-}$ mice exhibited increased fatty acid oxidation, increased energy expenditure and improved insulin sensitivity through AKT2 stimulation (108). The potential benefits arising from decreased malonyl-CoA levels to promote fatty acid oxidation and reduce body mass to increase insulin sensitivity prompted the development of a muscle specific deletion of ACC2 in mice. The tissue specific model produced mice that exhibited no protection from obesity or insulin sensitivity compared to the whole body $ACC2^{-/-}$ predecessor (83). Over-expression of MCD in the liver of rats fed a high fat

diet improved whole body insulin resistance (73). Conversely, the loss of whole body MCD resulted in resistance to diet-induced glucose intolerance, in the presence of increased skeletal muscle triacylglycerol and long chain fatty acids (107, 109). A study using human cultured skeletal myotubes investigated the effects of shifting substrate oxidation from lipid to glucose via RNA interference-mediated (siRNA) gene silencing of MCD under basal and insulin-stimulated conditions (106). Reducing MCD expression in human myotubes, lead to decreased lipid oxidation of palmitate with a rise in glucose oxidation under insulin stimulation. However, these studies are confounded by the loss of mitochondrial MCD and the subsequent increase of malonate, a cytotoxic metabolite that inhibits succinate dehydrogenase (110-114).

The interconnected nature of metabolic pathways, coupled with the redundancy and compensatory effects often seen by over-expression and knockout studies make it difficult to tease out the contributions of individual pathways to the pathophysiology of insulin resistance in skeletal muscle. Chemical-genetic techniques have been developed to acutely alter metabolic pathways in a manner that is temporal, cell-specific, and reversible (51, 52, 55). We have previously shown the posttranslational regulation of MCD in transgenic mice in a tissue specific manner via a biologically inert small molecule, Sheild-1 (55). Here, we acutely induced MCD in the skeletal muscle of obese and glucose intolerant mice to determine the impact of modulating skeletal muscle fatty acid oxidation in a model of diet-induced obesity. A two week induction of MCD in skeletal muscle did not alter body weight or ameliorate glucose intolerance, conversely it further impaired insulin signaling in the skeletal muscle of diet induced obese mice.

Surprisingly, an acute induction of MCD led to suppression of fatty acid oxidative genes suggesting a redundant and metabolite driven regulation of gene expression.

RESULTS

In Vivo Chemical-Genetic Regulation of Malonyl-CoA Decarboxylase in Skeletal Muscle.

Lipids mediate insulin resistance in skeletal muscle via an unknown mechanism; however, promoting the rate of fatty acid oxidation in skeletal muscle has been proposed to effect insulin sensitivity in this tissue (81, 108, 115-119). Given the importance of MCD in regulating skeletal muscle fatty acid oxidation, we generated transgenic mice where MCD (Tg-fMCD) can be regulated in a cell and chemical specific manner in order to determine the effect of acutely altering fatty acid metabolism in insulin resistance (55, 81). A cytoplasmic targeted MCD fused to a destabilization domain was cloned downstream of a lox mCherry stop cassette. Therefore, the expression of the transgene is controlled in a Cre-recombinase dependent manner to target transgene expression. The destabilization domain was derivatized from FKBP12 (FK506 binding protein 12) enabling reversible and dose dependent protein stabilization from the synthetic ligand Shield-1 (51). In order to target the transgene to skeletal muscle, Tg-fMCD mice were bred to mice expressing Cre recombinase from the human alpha skeletal muscle actin promoter, ACTA1, producing Tg-fMCD^{Skel} mice. Thus, we generated mice expressing cytoplasmic MCD that can be stabilized by Shield-1 in a skeletal muscle specific manner (**Fig. 1A**).

In order to determine the appropriate dose to achieve effective transgene stabilization we injected Tg-fMCD^{Skel} mice with increasing concentrations of Shield-1 in corn oil to determine basic in vivo pharmacokinetics and determine the required dose to effectively increase MCD. Shield-1 induced effective transgene stabilization at 20mg/kg (**Fig. 1B**). We had previously used corn oil to deliver Shield-1 to mice (55). However, since our goal was to determine the effect of lipid metabolism on insulin sensitivity, we chose a vehicle that did not contain dietary lipids. Others have reported in vivo delivery of Shield-1 in PEG/Tween/NNMDA (52). To prevent confounding effects of corn oil, we administered Shield-1 at varying doses in PEG/Tween/NNMDA. Injection of 60 mg/kg Shield-1 with a PEG/Tween/NNMDA effectively stabilized the MCD transgene (**Fig. 1C**). Therefore we chose 60 mg/kg Shield-1 in a PEG/Tween/NNMDA vehicle to alter MCD in mice.

A Two Week Induction of MLYCD in Skeletal Muscle Did Not Alter Body Weight or Glucose Sensitivity.

To assess whether increased fatty acid oxidation is associated with improvements in body weight and insulin sensitivity in a model of diet induced obesity, we made Tg-fMCD^{Skel} mice and littermate controls obese and insulin resistant with 12 weeks of high fat diet (HFD) (60% kcal from fat) feeding. An additional group of Tg-fMCD^{Skel} mice and littermate controls were fed a low fat diet (LFD) (10% kcal from fat) at 7 weeks of age for 12 weeks (**Fig. 2A**). Tg-fMCD^{Skel} mice and control mice on the high fat diet rapidly gained weight, and there was no difference between genotypes (**Fig 2B**). Mice on the LFD remained lean throughout the period (**Fig 2B**). Glucose sensitivity was

examined by preforming glucose and insulin tolerance tests. High fat diet-induced glucose intolerance was clearly evident in both genotypes (**Fig. 2 C,D**). Tg-fMCD^{Skel} mice and control mice began with elevated fasting blood glucose levels in the insulin tolerance test and failed to clear blood glucose as efficiently as their lean counterparts on the LFD (**Fig. 2E**). Thus, as expected, Tg-fMCD^{Skel} mice in the absence of the inducing ligand displayed no protection from obesity or insulin resistance.

To gain insight into the role of MCD in regulating metabolic dysfunction in the skeletal muscle, we treated Tg-fMCD^{Skel} and control HFD-induced obese and insulin resistant mice with Shield-1 (60 mg/kg) or vehicle every 48 hours for 2 weeks. Acute 2 week Shield-1 treatment did not alter body weight in Tg-fMCD^{Skel} or WT HFD mice (**Fig. 3A**). Moreover, MCD induction did not improve glucose sensitivity of these mice (**Fig. 3B**). Insulin measurements from Tg-fMCD^{Skel} HFD Shield-1 treated mice taken during the glucose tolerance test, show no difference in insulin sensitivity compared to WT HFD Shield-1 controls; both groups of mice remained insulin resistant compared to lean controls. These data suggest that acute induction of MCD in the skeletal muscle is not sufficient to alter adiposity or insulin sensitivity.

Acute Induction of MLYCD in Skeletal Muscle Repressed AKT Signaling.

To address possible tissue specific insulin sensitivity in the skeletal muscle, we performed an *in vivo* insulin stimulation followed by tissue collection. HFD fed Tg-fMCD^{Skel} and WT mice received an acute treatment of Shield-1 at 60 mg/kg every 24 hours for 5 days. Mice were injected with insulin 24 hours after the last dose of Shield-1 and gastrocenemius muscle was harvested 10 minutes after the insulin injection. As

expected insulin stimulated phosphorylation of AKT Ser ^{473} was decreased in Tg-fMCD^{Skel} HFD and control HFD mice compared to lean LFD controls (**Fig 4A**). Tg-fMCD^{Skel} HFD Shield-1 mice, compared to WT HFD Shield-1 controls, showed a further 2-fold suppression in insulin stimulated phosphorylated AKT Ser ^{473} relative to total AKT in the skeletal muscle (**Fig. 4A**). To determine the specificity of Shield-1 for the FY-MCD transgene in the skeletal muscle we showed expression and stabilization of the FY-MCD transgene by Shield- 1 only in the muscle (**Fig. 4A),** not in the liver, heart, or pancreas of the transgenic mice (**Fig. 5A-C).** These data suggest that induction of MCD in HFD induced insulin resistant skeletal muscle decreased insulin stimulation of AKT Ser ^{473,} phosphorylation and this exacerbates insulin resistance.

Induction of MLYCD Suppresses Fatty Acid Oxidation Genes.

Previously, we showed that an acute induction of MCD leads to an increase in fatty acid oxidation *in vivo* (55). Because malonyl-CoA and MCD are major regulators of skeletal muscle fatty acid oxidation, we examined the effect of inducing MCD on genes in the fatty acid oxidation pathway during the development of diet induced obesity and diabetes. Surprisingly, Tg-fMCD^{Skel} HFD Shield-1 mice, compared to WT HFD Shield-1 treated counterparts, had approximately 2-fold reduced protein abundance of CPT1B and oxidative phosphorylation complex proteins (**Fig 4A**). Due to reduced fatty acid oxidation protein expression, we questioned the possible activation of the energy sensor, AMPK. AMPK phosphorylation at Thr 172, the major activating phosphorylation site was not changed by MCD induction (**Fig. 4B**). Moreover, MCD induction did not alter phosphorylation of ACC, a target of AMPK. These data suggest that AMPK and ACC do

not play a role in mediating the decrease in insulin sensitivity observed in Tg-fMCD^{Skel} mice (**Fig. 4A**).

Because we observed a decrease in CPT1B and components of oxidative phosphorylation complexes 2, 3, and 4, suggesting a decreased capacity for oxidation, we determined if the decrease was due to regulation at the transcriptional level. Gene expression analysis of gastrocenemius muscle from Tg-fMCD^{Skel} HFD Shield-1 treated mice demonstrate a consistent decrease in genes involved in fatty acid oxidation compared to vehicle treated HFD controls (**Fig. 6**). These data demonstrate that an acute increase in skeletal muscle lipid oxidation by MCD in a model of obesity induces a concomitant reduction in the protein and mRNA abundance of of fatty acid oxidation genes.

DISCUSSION

A growing number of studies have shown a detrimental effect of skeletal muscle mitochondrial fatty acid oxidation in diet induced insulin resistance and obesity. We have increased MCD to increase fatty acid oxidation in skeletal muscle. Here, we demonstrate that 1) induction of MCD did not lead to changes in body weight in HFD induced obese mice, 2) an acute induction of MCD augmented skeletal muscle insulin sensitivity in HFD induced obese mice, 3) MCD induction resulted in a suppression of oxidative genes within skeletal muscle. The induction of MCD in the skeletal muscle exacerbated the diabetic phenotype by negatively effecting insulin/AKT signaling. These data provide insight into the pathophysiology of skeletal muscle insulin resistance and shows that

inducing MCD to increase fatty acid oxidation does not reverse obesity-induced glucose intolerance or adiposity.

The role of skeletal muscle mitochondria in the promotion or protection from metabolic dysfunction is not well understood. Based on the strong correlation between increased lipid consumption and insulin resistance, some have suggested that the accumulation of cytoplasmic lipid intermediates that are often seen in diabetic patients and animal models directly impairs insulin signaling (102, 120). Based on this, and the observation that a sedentary lifestyle promotes metabolic dsyfunction, it has been suggested that the stimulation of fatty acid oxidation could lower the concentration of toxic intermediates to improve insulin sensitivity by removing the lipid substrates. In support of this, individuals with type 2 diabetes and even prediabetes have decreased mitochondrial function (96, 101, 121, 122). However, several mouse models with increased lipid oxidation do not have improved insulin sensitivity (104). Inversely, metformin, which is widely used to treat diabetic patients, has been proposed to work as a mild electron transport inhibitor (123-125) and mice with decreased fatty acid oxidation due to mutations in complex I of the electron transport chain are protected from diet induced glucose intolerance. In support of these findings, we were unable to observe improvements in body weight or insulin sensitivity by increasing fatty acid oxidation with the overexpression of MCD in our model of high fat diet induced insulin resistance.

Obesity induced insulin resistance is associated with reductions in fatty oxidation genes and mitochondrial dysfunction (126, 127). Human studies support the observation of decreased transcriptional control of fatty acid oxidative genes in the skeletal muscle of obese, highly insulin resistant people. Specifically, individuals in the most insulin

resistant and insulin sensitive groups had lowered expression of PGC1α, PPARα, and CPT1B (128). A second study described decreased mRNA content in PDK4, PGC1α, and PPARα in obese, but not lean individuals (129). In contrast, small molecule inhibition of CPT1 improved insulin sensitivity and increased pyruvate dehydrogenase activity to promote glucose oxidation, and AKT phosphorylation (130). These studies suggest the skeletal muscle employs different mechanisms to adapt to varying degrees of insulin resistance. The skeletal muscle may use alternate mechanisms to regulate macronutrient substrate switching to increase glucose oxidation in a prolonged state of over nutrition. (109, 131).

Induction of MCD herein indicates that increasing lipid oxidation in the skeletal muscle may be a contributing factor in diabetes. Skeletal muscle MCD regulates the concentration of malonyl-CoA, the precursor for fatty acid synthesis and elongation. Decreasing the concentration of malonyl-CoA, dysinhibits CPT1, the rate-limiting enzyme in mitochondrial fatty acid oxidation. Interestingly, acute MCD expression in the skeletal muscle down-regulated CPT1B and other genes in the lipid oxidation pathway at the transcriptional and protein level (Fig. 5-6). These fatty acid oxidation genes are known targets of PPARα transcriptional activation, suggesting a novel mechanism linking intermediary metabolism to PPARα transcriptional regulation. PPARα has been shown to play an important role in the transcriptional regulation of lipid and glucose metabolism, particularly in skeletal muscle fatty acid oxidation (132). Despite the importance of PPARα in guiding human physiology and disease, the identities of natural PPAR ligands remain unclear. Metabolites likely play larger roles in regulating genes and pathways that have not been appreciated yet. We suggest a possible mechanism where

increasing MCD and resulting increased acetyl-CoA and fatty acid intermediates generates a metabolic signal that has downstream effects on PPARα mediated transcriptional control.

Chemical genetics has the potential to unravel metabolite and pathway contributions to animal physiology and pathophysiology with a higher resolution because it combines the stringency of genetics with the quick kinetics and dose dependence of small molecule pharmacology. Taking a chemical genetic approach in the design of the MCD transgene affords several built in controls to regulate *in vivo* metabolism with the benefit of small molecule inhibition and genetic specificity. The MCD transgene allows direct visualization of key internal features to control for recombination, localization of transgene expression, and transgene activation by Shield-1 (55). The specificity and mechanism of action of Shield-1 for the destabilization domain is well defined and any off target effects can be controlled for in Shield-1 treated nontransgenic mice. (51). This approach avoids many of the pitfalls of chemical inhibitors whose specificity is rarely tested.

CONCLUSIONS

These results indicate that induction of MCD leads to decreased fatty acid oxidation gene expression, impairs skeletal muscle insulin sensitivity, and implicates PPARα in a fatty acid induced down regulation of oxidative genes *in viv*o to regulate the muscle's adaptive response to diet induced obesity and insulin resistance.

ACKNOWLEDGEMENTS

This work was supported in part by the American Heart Association (SDG2310008 to M.J.W.) and NIH NINDS (NS072241 to M.J.W.).

EXPERIMENTAL PROCEDURES

Antibodies and Chemicals- Rabbit polyclonal antibodies that recognize phospho-AKT (Ser 473), Pan AKT, phospho AMPK Thr 172, AMPKα, phosph ACC Ser 79, Acetyl-CoA Carboxylase were obtained from Cell Signaling Technology. Rabbit polyclonal antibody detecting endogenous MCD was obtained from Abcam. A polyclonal antibody for dsRED was obtained from Clontech. Stabilization of Shield-1 was confirmed with a rabbit polyclonal antibody for FKBP-12 (Thermo Scientific). MitoProfile total OXPHOS Rodent WB Antibody cocktail was obtain from MitoSciences. Alpha-Tubulin protein loading control was obtained from Sigma. Gastrocnemius muscle for tissue analysis of signaling proteins, detection of endogenous recombination markers, and stabilization by Shield-1 was harvested and immediately flash frozen in liquid nitrogen. Total protein was extracted by tissue homogenization in cold lysis buffer consisting of 50 mM Tris-HCl, 150 nM NaCl, 1mM EDTA, 1% Triton X-100, with protease and PhosStop phosphatase inhibitor cocktail (Roche). Tissue homogenates were pelleted at 16,000 *g* for 30 minutes at 4°C and supernatants collected into fresh, cold micro centrifuge tubes. Protein estimation by Pierce BCA Protein Assay Kit was used to determine protein concentration in supernatants. Proteins were separated using NuPAGE Novex 4-12% Bis-Tris Gels in NuPAGE MOPS SDS running buffer. Proteins were transferred to PVDF membranes (0.45 μm), blocked in 5% non-fat milk and detected by immunoblotting with the antibodies above. HRP-conjugated secondary antibodies were detected using Amersham ECL Prime Western Blotting Detection Reagent (GE Healthcare) and detected using the FluorChem Western Blot imaging system (Cell Biosciences). Shield-1 was synthesized as previously reported (51, 74). Shield-1 was dried under a stream of nitrogen gas and

reconstituted in 50% *N,N*-dimethyacetamide and 50% of a 9:1 PEG-400:Tween-80 mixture (52). Shield-1 was administered intraperitoneally.

Animal studies- Tg-fMCD mice were bred to mice expressing Cre from a muscle specific (human alpha-skeletal actin) promoter obtained from Jackson Laboratory to generate Tg-fMCD^{skel} mice (78). Tg-fMCD^{skel} and wildtype littermates were maintained on a standard chow diet, with free access to food and water and maintained on a 12 hour light-dark photocycle in a temperature controlled environment. At 6 weeks of age, Tg-fMCD^{skel} and control littermate male mice were transitioned from a standard chow diet to a 60% kcal from fat high fat diet (HFD) (D12492, Research Diets, Inc.) or 10% kcal from fat low fat diet (LFD) (D12450J, Research Diets, Inc.). Body weights were measured weekly. Onset of glucose intolerance was assessed by glucose and insulin tolerance tests. At 19 weeks of age, Tg-fMCD^{skel} and control mice were injected i.p. with 60mg/kg Shield-1 (40 µl formulated in 50% N,N-dimethylacteamide and 50% of a 9:1 PEG-400:Tween-80 mixture) or vehicle alone. Mice received Shield-1 or vehicle injections every 48 hours for 2 weeks. Glucose tolerance tests were repeated on mice to measure efficacy of Shield-1 treatment to lower fasting blood glucose and increase insulin sensitivity. Mice used for the insulin stimulation studies, were i.p. injected with 60mg/ kg Shield-1 or vehicle every 24 hours for 5 days before the stimulation. Acute insulin stimulation was performed on mice following a 6 hour fast. Mice were injected i.p. with a 1U/ kg insulin (Sigma, bovine pancreas) dose. Tissues were collected 10 minutes after insulin injection, frozen in liquid nitrogen, and stored at -80°C. Animal experiments were done in accordance with the National Institutes of Health Guide for the Care and

Use of Laboratory Animals and under the approval of the Johns Hopkins Medical School Animal Care and Use Committee.

Glucose and Insulin Tolerance Testing –Mice were fasted for 6 hours before i.p. injection with 1.25 mg/g glucose or 0.8 U/kg of insulin (Sigma) in a 0.9% NaCl solution. Blood glucose was assayed from tail blood at times, 0, 15 min, 30 min, 60 min, 120 min for the GTT after glucose injection. Blood glucose was assayed from tail blood at times, 0, 15 min, 30 min, 60 min after the insulin injection for the ITT. Serum insulin was collected at the 15 minute time point during the GTT and measured using a mouse insulin ELISA kit (Millipore).

Quantitative real time PCR analysis- - Isolated Gastrocnemius muscles from Tg-fMCD^{skel} and control male mice were frozen in liquid nitrogen and stored at -80°C until homogenization with Trizol (Life Technologies) to isolate RNA. The conversion of RNA to cDNA, was performed by using a high capacity cDNA reverse transcription kit (Applied Biosystems). The following PCR primer pairs were used for this study:

ACOT1 forward, 5'-GACAAGAAGAGCTTCATTCCCGTG-3′,

ACOT1 reverse, 5'-CATCAGCATAGAACTCGCTCTTCC-3′,

CPT1B forward, 5′- GGTCCCATAAGAAACAAGACCTCC-3′,

CPTIB reverse, 5′- CAGAAAGTACCTCAGCCAGGAAAG-3′,

MCAD forward, 5′-GTTGAACTCGCTAGGCTCAGTTAC-3′,

MCAD reverse, 5′-CTCTGTGTTGAATCCATAGCCTCC-3′,

PDK4 forward, 5′-AGGGAGGTCGAGCTGTTCTC-3′,

PDK4 reverse, 5′- GGAGTGTTCACTAAGCGGTCA-3′,

PPARalpha forward, 5'-ACAAGGCCTCAGGGTACCA-*3′,*

PPARalpha reverse, 5'-GCCGAAAGAAGCCCTTACAG-*3′,*

PGC1alpha forward, 5'-CAGCCTCTTTGCCCAGATCT-*3′*

PGC1alpha reverse, 5'-CCGCTAGCAAGTTTGCCTCA-*3′*

18S rRNA forward, 5′-GCAATTATTCCCCATGAACG-*3′*,

18s rRNA reverse, 5'-GGCCTCACTAAACCATCCAA -*3*,

Statistics- Statistical analyses were performed using Student's 2 tailed *t*-test. Significance is defined when $p<0.05$. Data is displayed as mean ± SEM.

FIGURES

Figure 3-1. Tissue specific chemically inducible Malonyl-CoA Decarboxylase. (**A**) Schematic diagram of MCD transgene. (**B**) Tg-fMCD^{skel} mice were injected i.p. with vehicle or Shield-1 at various doses. Samples were collected 24 hours after injection and muscle samples were blotted with the indicated antibodies. (**C**) Dose and delivery vehicle analysis of Shield-1 in Tg-fMCD^{skel} mice. Gastrocenemius muscles from i.p. injected mice with vehicle or Shield-1 at varying doses and delivery methods were collected 24 hours post treatment to determine efficacy of Shield-1 stabilization by western blot for FKBP12.

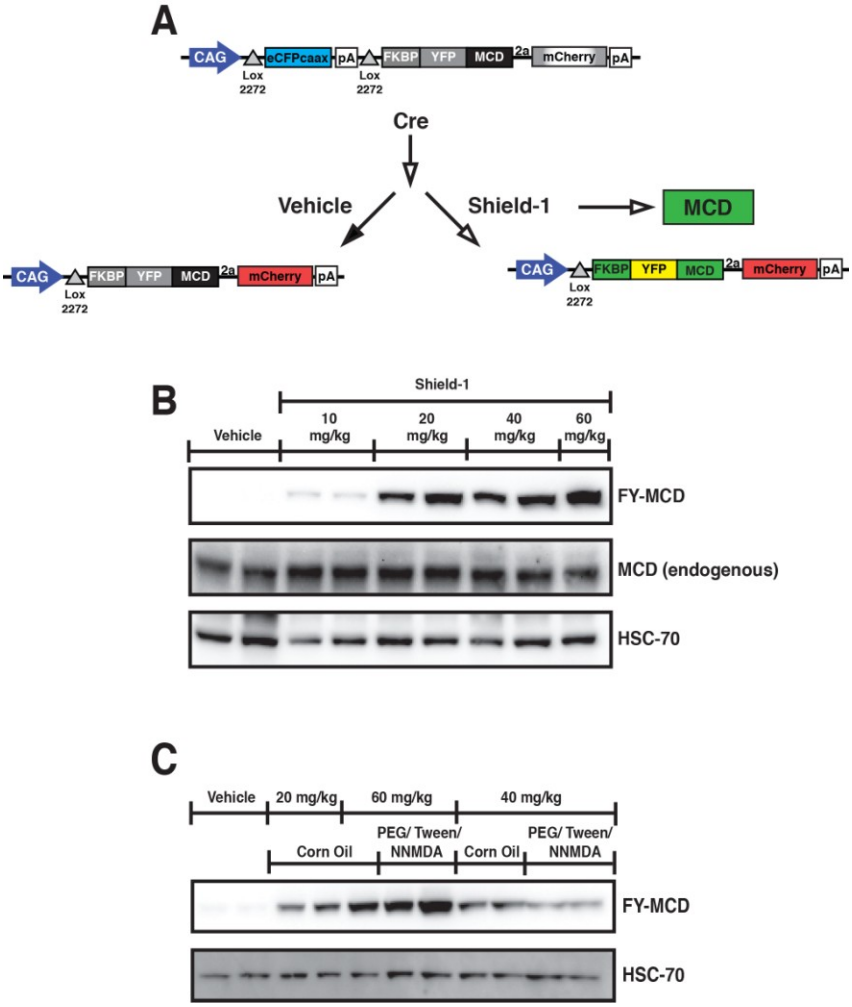


Figure 3-2. The Development of Diet Induced Obesity and Insulin Resistance in Tg-fMCD^{skel} mice. (**A**) Timeline of model for Control and Tg-fMCD^{skel} mice placed on low fat diet (LFD) or high fat diet (HFD) followed by treatment of Shield-1 to induce MLYCD within the skeletal muscle. (**B**) Mice were weighed weekly to monitor body weight gain. (**C**) Confirmation of glucose intolerance by glucose tolerance test (GTT) and (**D**) insulin in control and Tg-fMCD^{skel} mice. (**E**) Insulin tolerance test (ITT) confirms insulin resistance in mice fed the high fat diet.

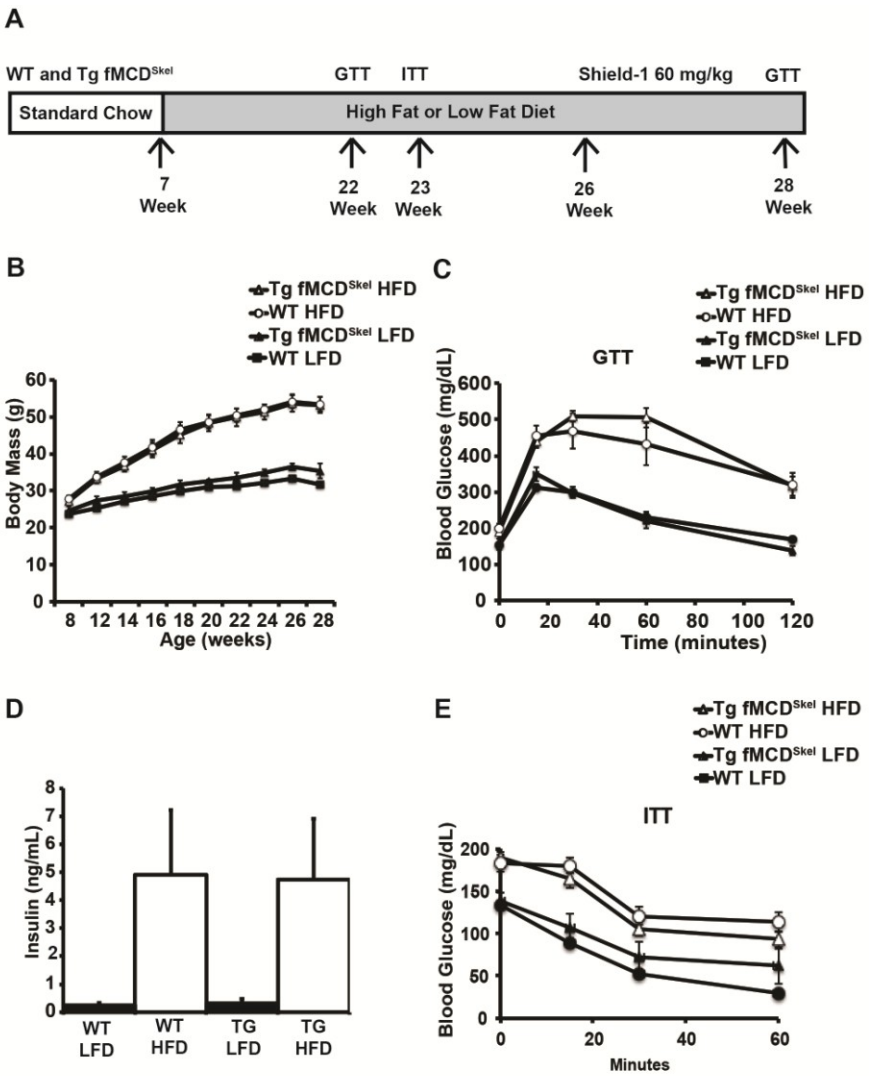


Figure 3-3. Acute Induction of MCD Does Not Reverse Diet Induced Obesity or Insulin Resistance. Tg-fMCD^{skel} and control mice on HFD and LFD received 8 doses of Shield-1 (60 mg/kg) or vehicle control. (**A**) Body weights were measured before and after Shield-1 treatment. (**B**) Mice were subjected to a GTT to determine glucose sensitivity.

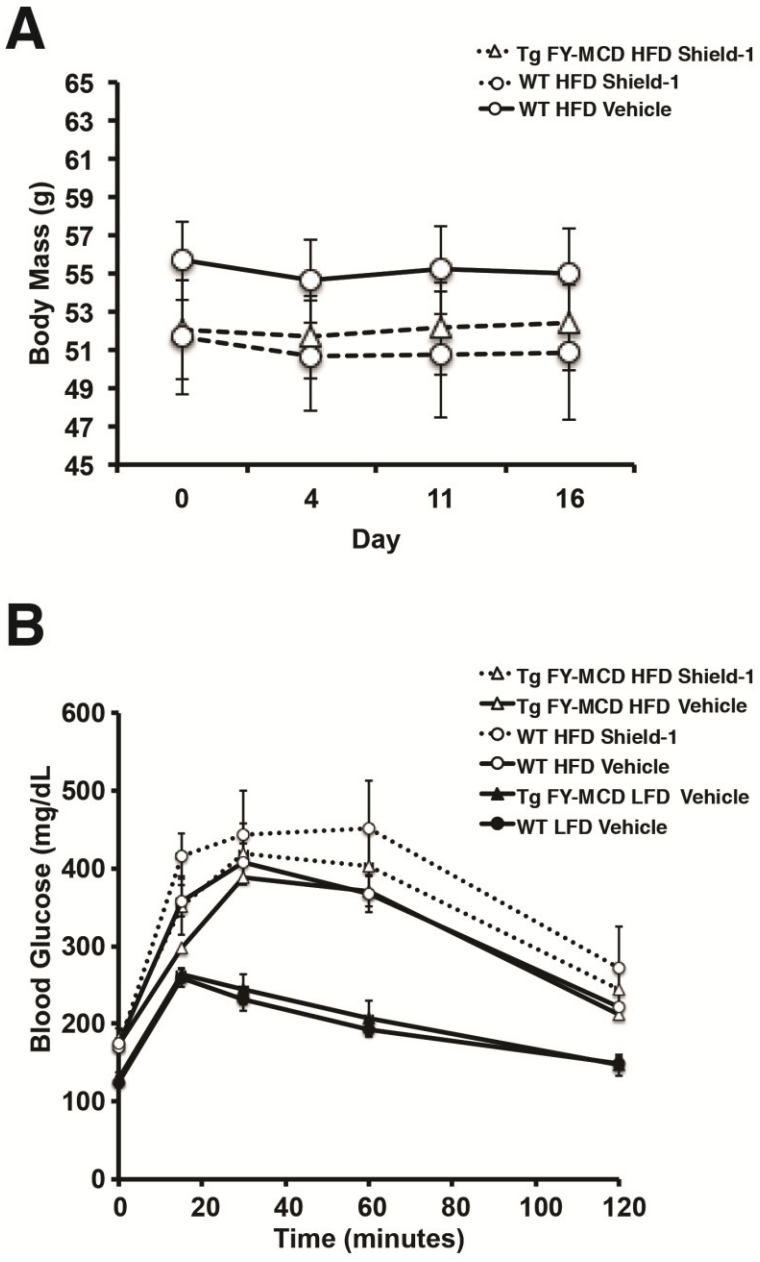


Figure 3-4. MCD induction suppresses AKT signaling and fatty acid oxidation. Western blot of gastrocenemius muscle isolated from Tg-fMCD^{skel} and control mice on LFD or HFD given Shield-1 (60mg/kg) or vehicle for 5 days. Mice were fasted for 6 hours before an insulin stimulation. (**A**) AKT Ser 473 phosphorylation, and ACC phosphorylation at Ser79 are normalized to total protein and quantified. CPT1B and OXPHOS complexes are normalized to alpha Tubulin and quantified. Samples were blotted with FKBP12 (for transgene stabilization), MLYCD (endogenous expression), mCherry (transgene expression) and alpha Tubulin (loading control). (**B**) AMPK Thr 172 phophorylation was determined, normalized for total AMPK and quantified. (**C**) Endogenous levels of MLYCD were determined, normalized to alpha Tubulin, and quantified. Results are expressed as *p< 0.05 compared to WT HFD Shield. Data represent mean ± SEM.

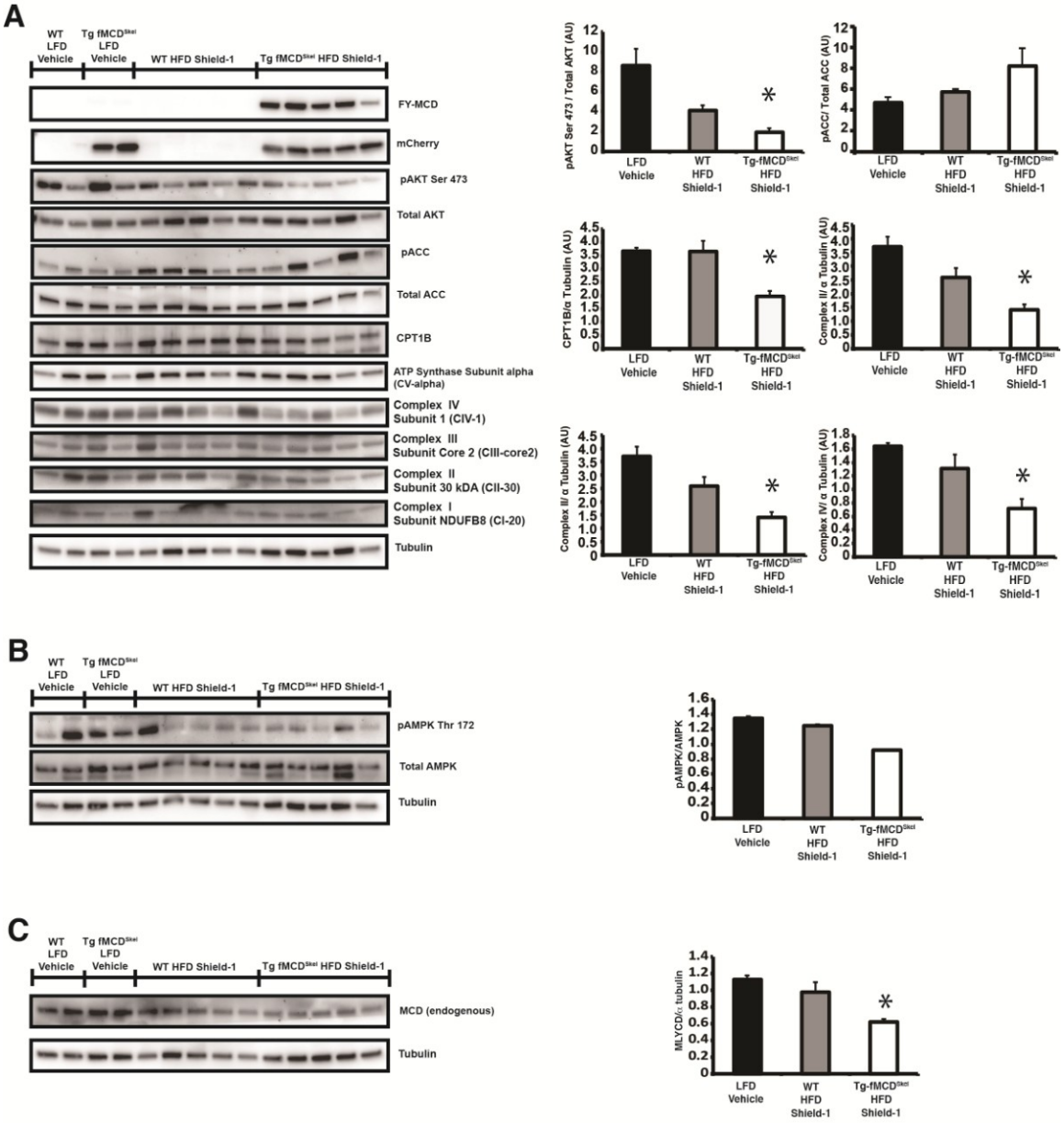


Figure 3-5. MCD transgene expression and Shield-1 stabilization is specific to skeletal muscle. Western blot of liver (**A**) pancreas (**B**) and heart (**C**) tissue extracted from Tg-fMCD^{skel} and control mice on HFD and LFD treated with Shield-1 (5 doses at 60 mg/kg) or vehicle. Samples were blotted for FKBP12 (for transgene stabilization), MLYCD (endogenous expression), mCherry (transgene expression), alpha Tubulin (loading control). Positive controls for mCherry and FKBP are derived from skeletal muscle of Tg-fMCD^{skel} mice treated with vehicle or Shield-1.

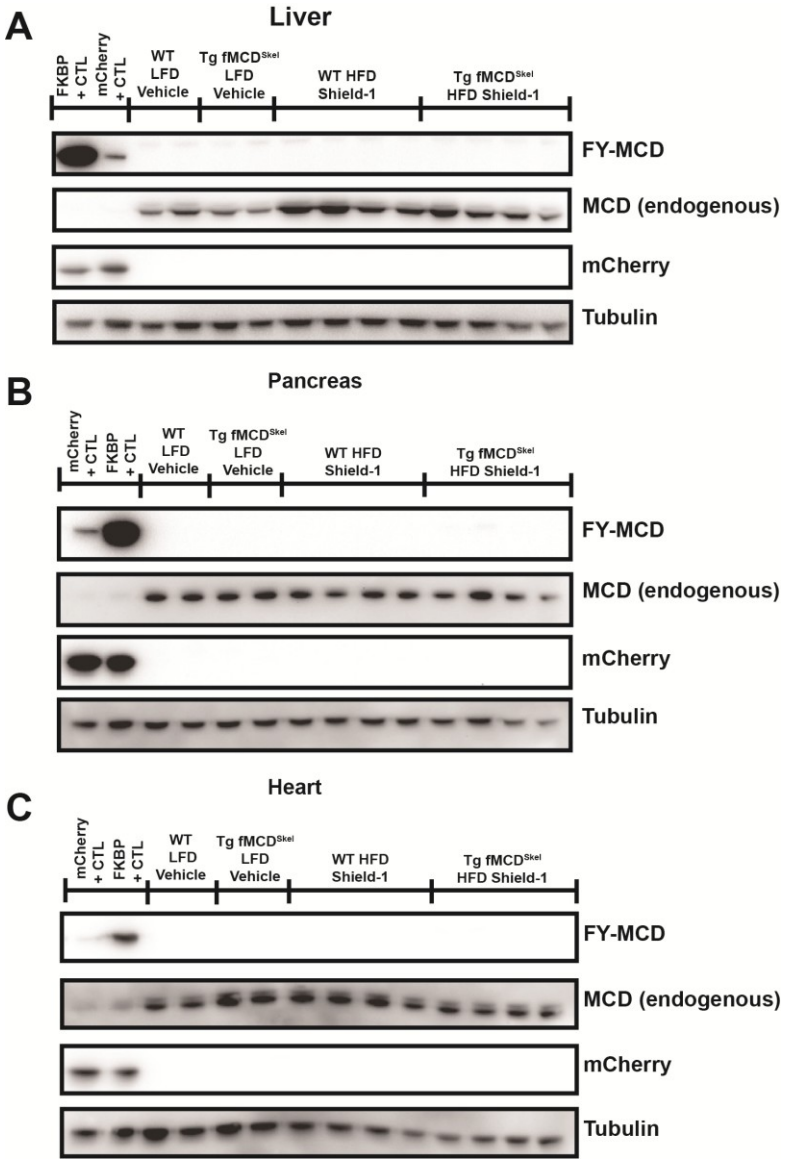
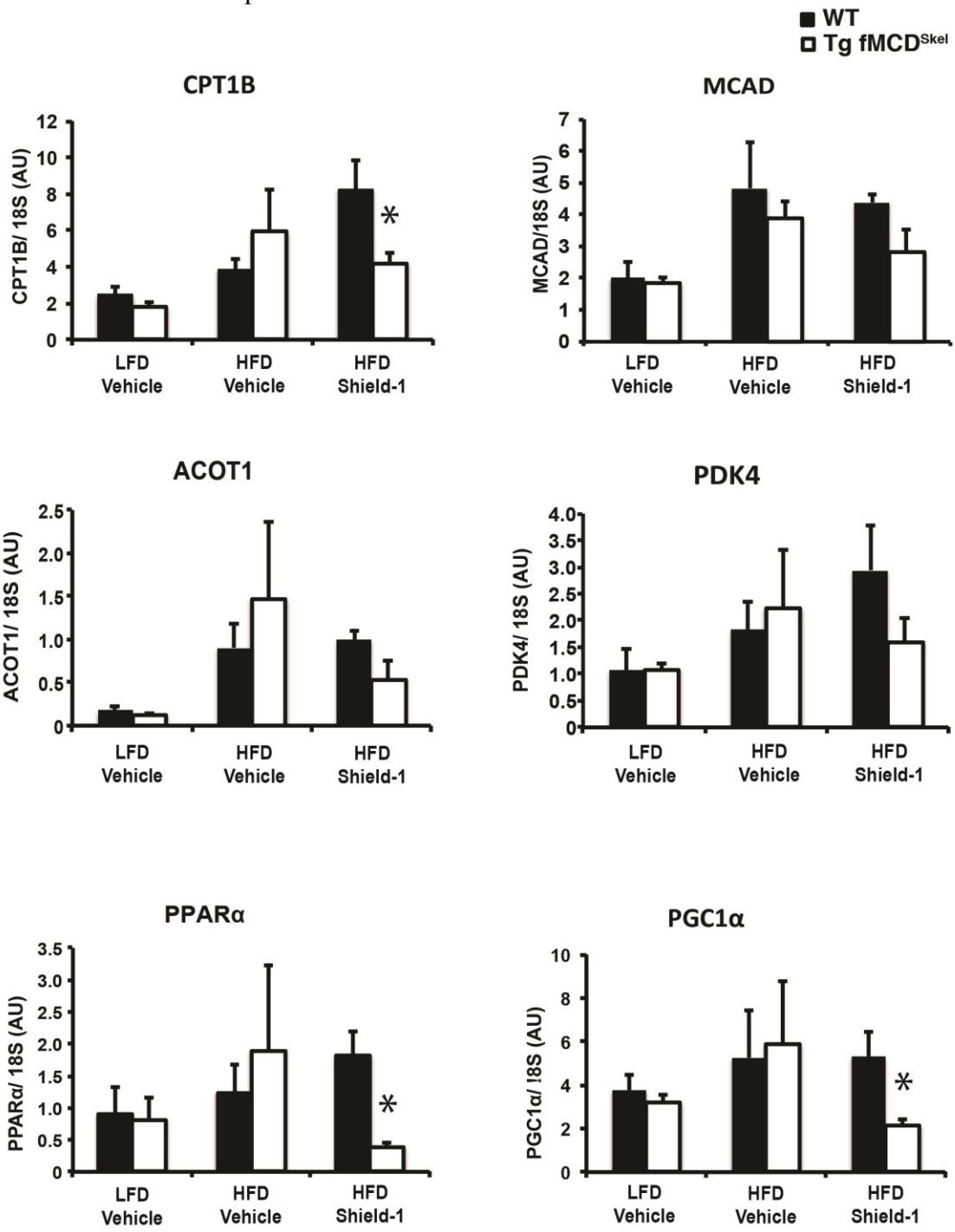


Figure 3-6. Fatty Acid Oxidation genes are down-regulated upon induction of MCD. qPCR analysis of key fatty acid oxidation genes, CPT1B, MCAD, ACOT1, PDK4 and transcriptional regulators of lipid oxidation, PPARα PGC1α. * $p < 0.05$ compared to WT Shield-1 treated. Data represent mean ± SEM.



CHEMICAL GENETIC REGULATION OF MALONYL-COA DECARBOXYLASE IN SKELETAL MUSCLE INSULIN RESISTANCE

CHAPTER IV: Synthesis

OVERVIEW OF RESEARCH FINDINGS

In this thesis, a simple and broadly applicable method for regulating protein and pathway function *in vivo* has been developed. This method combines mouse conditional transgenics and synthetic posttranslational protein stabilization to produce mice that have dually regulated enzyme expression. The conditional KO of genes in mice has lead to a revolution in the understanding of gene function *in vivo*. Small molecule inhibition of specific proteins would be better for understanding protein function in adult animals. However, some proteins have no such inhibitors and mostly all small molecule inhibitors have off target effects that cannot be controlled for without an existing KO mouse to test the compounds against. Allele-specific small molecules can be made to selectively and potently alter protein function by allowing the WT allele to be used in control experiments for specificity. The targeted chemical-genetic method developed in this thesis allows the study of unknown genes, enzymes where the reaction mechanism is not known, structurally similar protein families or proteins where small molecule inhibitors are in demand, but not available. Therefore, new methodology is needed and important for deconstructing metabolic pathways that are highly interdependent and cross-regulated. Our method provides a practical, specific, and reversible means of manipulating metabolic pathways in adult mice to provide biological insight.

In order to produce dose dependent, small molecule-regulated posttranslational stabilization to study metabolites in vivo, we used a synthetic posttranslational protein stabilization system developed by Tom Wandless' lab. Wandless constructed a modified FK506 binding protein 12 (FKBP12) that binds to and is reversibly stabilized by a synthetic, biologically inert small molecule, Shield-1. Several features were added to the

FKBP12/Shield-1 system to create internal controls for transgene insertion, recombination, and stabilization. A picornavirus 2A peptide-linked mCherry was fused in-frame to enable a ratiometric, autonomous measurement of stabilization. The conditional protein stabilization domain (FKBP12) was placed after a floxed stop cassette to enable Cre recombinase mediated targeting of the regulated domain. Heterospecific lox sites to minimize cross-genomic recombination with loxp sites were used. All of the activities are linked to a specific fluorophore to enable direct visualization of transgene expression, CRE-mediated recombination and Shield-1 induced stabilization. This vector was used to regulate MCD protein and enzyme activity. MCD is an ideal candidate for posttranslational protein regulation because of its enzymatic regulation of its substrate, malonyl-CoA. Exerting control over malonyl-CoA through MCD has broad experimental utility because malonyl-CoA is situated at a critical node in fatty acid metabolism. Malonyl-CoA is the product of the rate- limiting step in de novo fatty acid synthesis and elongation, and allosterically inhibits the rate-setting step in fatty acid beta-oxidation. Regulation of malonyl-CoA by MCD has been shown to play a role in neurochemistry, cancer metabolism, obesity and diabetes.

Small molecule regulation of MCD was validated by several methods. Two HEK 293T stable cell lines were produced that expressed FKBP12-eYFP-MLYCD-2A-mCherry (FY-MLYCD) or FKBP12-eYFP-2A-mCherry (FYFP). These stables cell lines were used to characterize the system initially. Addition of Shield-1 induced FYFP and FY-MLYCD in a dose-dependent and reversible manner. Saturation of stability was achieved 12 hours post treatment and stable cells returned to basal levels 48 hours after Shield-1 removal. To demonstrate that the FY-MLYCD fusion retained enzymatic

activity, Shield-1 or vehicle was added to FY-MLYCD stable cells for 16 hours and then assayed for MCD activity. Shield-1 induced a 4-fold increase in MLYCD activity. These experiments show Shield-1 can regulate MCD protein levels and enzyme activity. The vector was modified to affect metabolism in live behaving mice. First, a lox2272 flanked eCFP cassette was cloned into a ubiquitous mammalian expression vector and the FY-MLYCD transgene was cloned downstream of the floxed stop cassette. This feature allowed FY-MLYCD to be produced only after CRE-mediated excisions of the eCFP stop cassette. Transgenic mice expressing Lox2272-eCFPcaax pA Lox2272-FKBP12-eYFP-MLYCD-2a-mCherry (Tg-fMCD) were produced and characterized for eCFP expression by Western blot and direct epifluorescent visualization in cryosections. CFP expression was the highest in the muscle, pancreas, and kidney, but not in the brain and liver. These mice were then crossed to mice that ubiquitously express a tamoxifen-inducible Cre recombinase, CRE^{ERT2} (77) to produce double-transgenic mice (Tg-$fMCD^{ERT2}$). Tg-$fMCD^{ERT2}$ animals were injected i.p with tamoxifen to induce recombination of the eCFPcaax domain in vivo. Shield-1 was administered to induce transgene expression of FY-MLYCD. Recombination was seen in vivo as the expression of untargeted mCherry, and stabilization was visualized by the expression of cytoplasmic eYFP. Shield-1 stabilized FY-MLYCD in tissues that express the transgene, including the pancreas, skeletal muscle, heart, and adipose tissue. Tamoxifine treated Tg-$fMCD^{ERT2}$ mice were given Shield-1 or vehicle and assayed for their ability to fully oxidize radiolabeled palmitate to CO_2. Shield-1 induced an ~3-fold increase in fatty acid oxidation, during the fed state, in Shield-1 treated Tg-$fMCD^{ERT2}$ mice, but not vehicle control treated Tg-$fMCD^{ERT2}$ mice. The system was tested in a tissue specific context by

crossing transgenic mice (Tg-fMCD) to mice expressing Cre recombinase from human alpha-skeletal muscle actin promoter to produce Tg-fMCD^{skel} mice. Administration of Shield-1 to Tg-fMCD^{skel} mice resulted in the induction of eYFP-MLYCD with increasing doses of Shield-1. These experiments show the utility of regulating MCD through a targeted chemical-genetic technique that allows rapid and reversible regulation of MCD protein and enzymatic activity in a tissue-specific and temporal manner to affect metabolic pathways.

In addition to demonstrating the power of targeted chemical-genetics in generating mice with dually regulated enzyme expression, I have used this approach to decipher the role of MCD in diet induced insulin resistance in the skeletal muscle. Investigative support for increasing lipid oxidation as a means to remove excess lipid deposition in the muscle, increase glucose sensitivity, and restore mitochondrial function has been a sought after method to improve metabolic health in diabetic patients. Studies using transgenic mouse models of tissue-targeted deletions or overexpression of genes involved in fatty acid metabolism have arrived to different conclusions on the benefits of increased fatty acid uptake in the skeletal muscle. In this thesis, it is established that acute overexpression of MCD in a model of skeletal muscle insulin resistance increases the severity of diet induced glucose intolerance. The evidence shows that acute overexpression of MCD by Shield-1 treatment did not improve insulin sensitivity as measured by GTT and decreased phosphorylation of AKT Ser^{473} in obese insulin resistant Tg-fMCD^{Skel} mice. Acute MCD overexpression by Shield-1 treatment did not change body weights of obese insulin resistant Tg-fMCD^{Skel} mice compared to their wiltype controls. Acute overexpression of MCD by Shield-1 treatment decreased protein abundance of

CPT1B, MCD, and components of oxidative phosphorylation complexes 2, 3, 4 in obese insulin resistant Tg-fMCD^{Skel} mice. The decrease in protein expression was due to transcriptional down-regulation of oxidative genes in a PPARα dependent manner. Combined, these results indicate that excessive MCD overexpression contributes to sustained metabolic dysfunction in obesity induced insulin resistance in the skeletal muscle.

DIRECTIONS FOR FUTURE RESEARCH

Would an exercise intervention improve skeletal muscle insulin resistance with Shield-1 mediated MCD overexpression?

The effects of increased fatty acid oxidation through MCD overexpression in our model proved more detrimental than beneficial. It is important to note that our transgenic mice continued to consume the high fat diet during the Shield-1 treatment periods with no opportunity to increase energy expenditure through exercise. It is unclear if an intervention of Shield-1 treatment to stimulate MCD overexpression in combination with treadmill exercise would stimulate a positive health outcome through weight loss and increased glucose sensitivity in Tg-fMCD^{skel} mice. By housing transgenic mice and their controls in an Oxymax/CLAMS (comprehensive laboratory animal monitoring system) with a treadmill, we can determine their food intake, body mass, activity levels, and use indirect calorimetry to determine their whole body substrate oxidation levels to assess changes in their diabetic phenotype after the exercise regime. To assess insulin sensitivity, glucose tolerance tests administered before and after exercise training are used to characterize improvements in glucose intolerance. To determine improvements in

insulin sensitivity from exercise, we would measure mRNA levels of PPARα and PGC1α in red (oxidative) and white (glycolytic) skeletal muscles. In the skeletal muscle, PGC1α has been shown to stimulate mitochondrial biogenesis and regulate genes involved in fatty acid catabolism through its coactivation with the PPARs and estrogen-related receptor α (ERRα). PGC1α is decreased by inactivity and chronic high fat feeding and increased in response to acute exercise (126, 133). PGC1α mediated remodeling of downstream metabolic pathways, such as the TCA cycle and electron transport chain will also be evaluated because human and animal studies link decreased PGC1α activity and the reduction of genes involved in oxidative phosphorylation to the development of type 2 diabetes (119, 133, 134). I predict exercise training will enhance the muscle's capacity to tolerate high levels of fatty acids through PPARα and PGC1α activation and increase insulin sensitivity.

Would a time course and dose treatment of Shield-1 alter insulin resistance in obesity induced insulin resistant Tg-fMCD^{skel}?

An observation presented in this thesis is that high fat diet induced insulin resistance is associated with decreased muscle fatty acid oxidation, transcriptional downregulation of PPARα and PGC1α, and reduced protein abundance of electron transport complexes. To better assess if MCD expression increases the severity of insulin resistance and PPARα repression, a time course and dose dependence of Shield-1 treatment should be performed. I predict a lower dose of Shield-1 will produce a milder form of insulin resistance during an acute (5 day), 2 week, or 1month extended Shield-1 treatment in Tg-fMCD^{skel} mice compared to wildtype obese controls. Tg-fMCD^{skel} mice treated with an extended time course of Shield-1 at 60mg/kg will remain diabetic with

transcriptional repression of PPARα and PGC1α and decreased expression of lipid oxidation genes.

Does MCD expression generate a ligand that negatively regulates the transcriptional activation of oxidative genes by PPARα in the skeletal muscle?

The PPARs belong to a family of nuclear hormone receptors that are activated by a variety of lipid-derived ligands. To this date, their natural ligands still remain a mystery. There are still many unanswered questions, including their identities, i.e., what are the concentrations required in the nucleus to trigger a PPAR-dependent transcriptional response? Are there several ligands working together to mediate this response? It would be important to identify the ligand that allows PPARα to respond to dietary stimuli to maintain energy homeostasis as the identification of PPAR ligands have important clinical implications in treating metabolic disease. To identify the potential ligand(s), a metabolomics approach using isolated muscle nuclear fractions from transgenic and control mice treated with Shield-1 or vehicle are compared. The data generated will be critical for understanding the function of PPARα and their use as potential drug targets in metabolic disease, such as diabetes. I predict pathways involved in neutral lipid synthesis are up regulated in response to increased fatty acid oxidation as a negative feedback mechanism to decrease oxidative gene expression as a protective mechanism to decrease fatty acid oxidation generating damaging oxidative byproducts.

Public Health Significance

Skeletal muscle plays an important role in maintaining insulin mediated glucose sensitivity and regulating fatty acid metabolism. Studies of obese, insulin resistant individual and diabetics have implicated dyslipidemia and intramuscular accumulation of fatty acid metabolites as core features for the development of these disease states. Understanding the mechanisms that the muscle uses to handle excess lipid intake, will further enhance our understanding to develop new treatments of obesity related diseases. Enhancing fatty acid oxidation has been proposed as a therapeutic measure to prevent the accumulation of lipotoxic intermediates that are deemed deleterious by acting on and inhibiting key regulators of the insulin-signaling pathway. MCD is a key enzyme responsible for regulating malonyl-CoA concentration and functions in the control of balancing lipid oxidation and glucose metabolism in the muscle. A whole body knock out of MCD protected against the development of diet induced insulin resistance, but the tissue specific mechanisms remain unresolved since MCD is expressed in multiple tissues that are responsible for maintaining blood glucose and energy homeostasis. Taking a chemical genetic approach to determine the role of skeletal muscle MCD and the subsequent preference for lipid oxidative pathways in a model of diet induced obesity and insulin resistance has demonstrated that, on the contrary, lipid oxidation foments the diabetic environment. The data presented in this dissertation provides novel evidence for the additional repression on insulin signaling and the transcriptional deactivation of PPARα to suppress the activation of oxidative genes through an unknown ligand functioning in a feed forward mechanism. Thus, therapeutic approaches aimed to

stimulate fatty acid oxidation will hinder the progress in finding novel treatments for combating metabolic disease.

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Research Experience

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Academic and Professional Honors

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2012	Invited speaker at the Keystone Symposia on the Genetic and Molecular Basis of Obesity and Body Weight Regulation, Feb 3, 2012, Santa Fe, New Mexico
2011	Keystone Symposia Underrepresented Minority Scholarship Travel Award

2006	Second place poster presentation at the Fifth Annual New England Science Symposium, sponsored by the Harvard Medical School and the Biomedical Science Careers Program
2005	FASEB MARC Travel Grant Award
2004	Western Student Medical Research Forum Endocrinology Subspecialty Award
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2004	First place oral presentation at the 18th Annual Undergraduate Research Symposium, Graduate School of Biomedical Sciences, University of Texas Medical Branch at Galveston
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Publications

Research Papers:
Rodriguez S, Wolfgang MJ. 2011. Targeted chemical-genetic metabolic regulation in vivo. Chemistry and Biology, 2012 Mar 19 (3): 391-398.

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Abstracts:

Rodriguez S, Wolfgang MJ. Targeted chemical-genetic metabolic regulation in vivo. Keystone Symposia on the Genetic and Molecular Basis of Obesity and Body Weight Regulation, Jan 29 - Feb 3, 2012, Santa Fe, New Mexico (Oral and poster presentation, Keystone Symposia Underrepresented Minority Scholarship Travel award).

Rodriguez S, Guo WH, Giraud J, and Lopez MF. IRS-2 Plays an Important Role in the Signaling of Igf-2 in Fetal Hepatocytes. 2006. Fifth Annual New England Science Symposium (sponsored by the Harvard Medical School and the Biomedical Science Careers Program), Boston, MA (poster presentation).

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Rodriguez S, Vera Y, V Pope, M Castanares, YH Lue, V Atienza, C Wang, RS Swerdloff and AP Sinha Hikim. 2004. Functional Role of Caspases in Heat-Induced Testicular Germ Cell Apoptosis. Western Student Medical Research Forum, Carmel, CA (oral presentation and Western Student Medical Research Forum Endocrinology Subspecialty Award).

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Rodriguez S, Vera Y, Lue YH, Diaz-Romero M, Wang C, Swerdloff RS, Sinha Hikim AP. 2003. Translocated Cytosolic DIABLO Through Interaction with XIAP

Regulates Heat-Induced Testicular Germ Cell Apoptosis in FAS Ligand-Defective Mice. Western Section AFMR, Carmel, CA (oral presentation).

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Hikim AP, Lue YH, **Rodriguez S**, Yen PH, Wang C, Swerdloff RS. Activation of the Mitochondria-Dependent Apoptotic Pathway in Heat-Induced Germ Cell Apoptosis in FAS Ligand-Defective Mice. 2002 Program of the 27th Annual Meeting of the American Society of Andrology, Seattle, Washington (oral presentation).

Rodriguez S, Lue YH, Yen PH, Swerdloff RS, and Sinha Hikim AP. 2002. Activation of the Mitochondria-Dependent Apoptotic Pathway in Heat-Induced Testicular Germ Cell Apoptosis in Mice.Western Student Medical Research Forum, Carmel, Ca (oral presentation).

Reviews:
Rodriguez S and Wolfgang MJ. Regulation by Fatty Acids/Malonyl-CoA in the Brain. Encyclopedia of Biological Chemistry 2nd Edition, Elsevier Inc., In Press. 2013

Manuscripts in Preparation:
Rodriguez S, Ellis J, Wolfgang MJ. 2014. In vivo Chemical-Genetic of Skeletal Muscle Malonyl-CoA Decarboxylase Does Not Improve Insulin Sensitivity. BMC Biochemistry.

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